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Biotransformation of Oxo-Bile Acids Derivatives by Human Gut Bacteria

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Biotransformation of Oxo-Bile Acids Derivatives By Human Gut Bacteria

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BY

Laina Sallam

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ABSTRACT

Bile acids are one group of steroids that can be metabolically transformed by human gut anaerobes. One of these transformations is the conversion of primary bile acids to secondary bile acids by 7α-dehydroxylating gut bacteria such as *Clostridium scindens, Clostridium hylemonae,* and *Clostridium hiranonis.* These anaerobes metabolize bile acid derivatives via 3α- and 7α-hydroxysteroid dehydrogenases (HSDH). Studies to date have not reported if these organisms are capable of converting 12-oxolithocholic acid (12-oxoLCA) to deoxycholic acid (DCA). The emphasis of this study was to determine the ability of *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 to reduce 12-oxoLCA to DCA during growth, to identify 12α-HSDH genes in, *C. scindens* and *C. hiranonis,* to express, purify, and characterize the 12α-HSDH gene product (protein) of *C. scindens.* These organisms were grown anaerobically at 37°C in brain heart infusion (BHI) broth. Bile acids, prepared in methanol, were added to BHI broth to a final concentration of 0.1 mM. Following growth, cultures were extracted with ethyl acetate; extracts were dried, re-suspended in methanol, and subjected to thin-layer chromatography (TLC) for the detection of bile acids. In addition, 12α-HSDH recombinant enzymes were characterized and purified by affinity chromatography using Strep-Tactin® resin. Kinetic constants (K_m, K_{cat}, V_{max}, and K_{cat}/K_m) were determined for the purified recombinant proteins for the forward and reverse directions. Catalytic efficiencies K_{cat}/K_m toward the reductive direction were between 2.8-3.5-fold greater than oxidative direction. However, V_{max} and K_{cat} in the oxidative direction were between 1.6-2.9-fold higher than the reductive direction. The 12α-HSDH recombinant enzyme from each strain was shown to reduce 12-oxoLCA to DCA. Moreover, these organisms were able to transform other oxo-bile acids during growth.

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1. LITERATURE REVIEW

The role of gut microbiota in human health. The role of the human microbiota has become widely appreciated, and the variation of bacterial types and the balance of some species are known to play a significant role in human health (1). The human gut contains over 1,000 species and 15,000 bacterial strains, all categorized under 9 phyla (2). Studies have shown that between individuals there are differences that often exist between three genera (types) of bacteria, *Bacteroides*, *Prevotella*, and *Ruminococcus* (3). Based on these types of dominant bacteria in each individual, people will be more or less likely to develop particular diseases(4, 5). Therefore, the composition of a person's microbiome can ultimately determine potential health problems (e.g., cancer, immunity, and bowel diseases) for the individual (6). These microbiomes create a beneficial relationship with the host by supporting gut homeostasis, developing the intestinal epithelium cells, protecting against pathogens, and supporting host immunity (7). All of these microbial occupations allow gut microbiota to regulate very essential and vital functions within the host physiology (7). It is important to know that bacterial populations dominate the microbiota in the human intestinal tract. Thus, it is critical to investigate the different factors that might influence the interaction of the gut bacteria and how they impact such host functions as immunity, mood, and bile acid metabolism (8). Anaerobic microorganisms are the main colonizers of the human gut. Consequently, most studies have been focused on Bacterial genera such as *Clostridium, Veillonella, Ruminococcus, Eubacterium, Bifidobacterium, Lactobacillus, Fusobacterium, Peptococcus* and *Peptostreptococcus*, as they have been considered to be the dominant gut microbes (Figure1), (9–11).

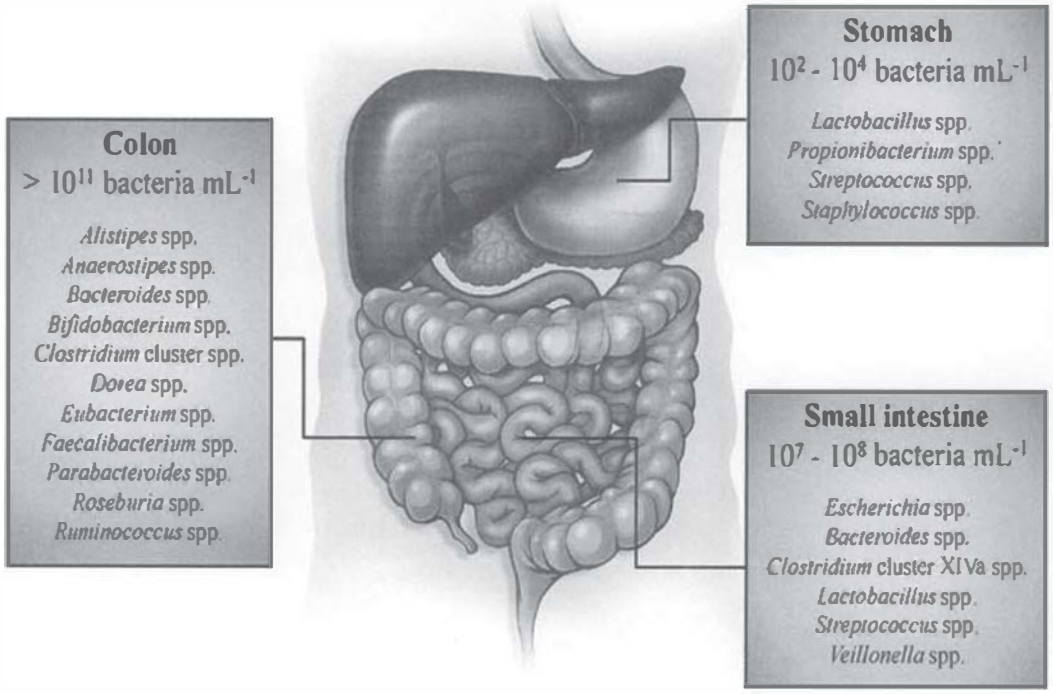


Fig 1. Diversity and populations of bacteria in different parts of the human gastrointestinal (GI) tract (58).

Bile acids chemistry. Bile acids, are defined as a group of steroids that synthesized in the liver via cholesterol and conjugated to the amino acid glycine and taurine, stored in the gallbladder and secreted into the lumen(12, 13). The conjugated bile acids are the major solutes in human bile (14). The synthesis of primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) multifaceted and requires about 17 individual enzymes, these enzymes occur in multiple intracellular parts that include the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes (13, 14). Before the primary bile acids are secreted into the canalicular lumen they are conjugated via an amide bond at the terminal carboxyl group with amino acids glycine or taurine (14). These conjugation reactions yield glycoconjugates and tauroconjugates, respectively (14). This conjugation process

increases the amphipathic nature of the bile acids making them more easily secret-able as well as less cytotoxic (14).

Large amounts of bile acids are secreted into the human intestine every day; about 95% of bile acids reach the duodenum and are absorbed across human intestinal and then recycled back to the liver(13). Only 5% continues to travel to the colon and is ultimately discharged through defecation(15). The bile acids not absorbed (5%) are subject to metabolism by the gut microbiota. One of the main reactions catalyzed by the gut microbiota is the transformation of primary bile acids CA and CDCA into secondary bile acids deoxycholic acid (DCA) and lithocholic acids (LCA), respectively. It is significant to indicate that cholecystokinin is the hormone that responsible for the secretion of the bile into the intestine, and this secretion is stimulated by the presence of fat in the duodenum (16). DCA absorbed in the colon enters enterohepatic circulation whereas LCA is insoluble and only a small amount of this secondary bile acid is reabsorbed (17, 18).

As a metabolic regulator, it has become clear that bile acids are also signaling molecules, the function of bile acids in regulating lipid and glucose homeostasis are appeared as ; i) bile acids and phospholipids solubilize cholesterol in the bile, which prevents the precipitation of cholesterol in the gallbladder; ii) bile acids facilitate the digestion of dietary triacylglycerols by acting as emulsifying agents that render fats accessible to pancreatic lipases; iii) enable the intestinal absorption of fat-soluble vitamins (14, 19).

Relative to the intra and extracellular activity of bile acids in the host, the binding of bile acids to FXRs attenuated the expression of many genes that are associated with bile acid homeostasis (14, 19). In addition to the roles of bile acids in lipid emulsification in the intestine and activating FXR, bile acids also participate in some signaling transduction

such as; i) activating c-JUN N- terminal kinase (JNK); ii) activate mitogen-activated protein kinase (MAPK) pathways are ligands for the G-protein-coupled receptor (GPCR) TGR5; and iii) activates nuclear hormone receptors such as farnesoid X receptor α (FXRα; NR1H4) (14, 20).

Bile acid toxicity. Bile acids are amphipathic molecules possessing both hydrophilic and hydrophobic parts (21). This explains how bile acids act as a detergent by solubilizing and transporting fat molecules for absorption (18). Bile acids also play a role in numerous physiological capacities, for example, processing and assimilation of lipids and lipid-solvent supplements in the intestinal lumen, control against bacterial excess, and disposal of cholesterol from the body (21). Relative to toxicity, bile acids can damage cellular DNA and lead to carcinogenesis, and DCA at high levels (~100 μM) can promote apoptosis in the epithelial cells (22). The continual damage of DNA will increase the mutation rate; hence, increased levels of bile acids are a major factor associated with pancreas and colon cancers in humans (17). Normally, during the physiological process, there is no metabolic pathway for the removal of the DCA from the bile acids pool. Toxicity of secondary bile acids is elevated by the dysbiosis of gut microbiota and that occurs when anaerobic bacteria such as, *C. scindens*, *C. hylemonae*, *C. hiranonis*, transformed many forms of oxo-bile acid derivatives to secondary forms (23). Consequently, high level of bile acids in the gut can suppress the growth of other commensal bacteria and boost the dysbiosis rate, that will lead to serious illnesses like metabolic syndrome, irritable bowel diseases, and cancers (17-18).

Diet and bile acids. The gut is the largest organ in the body, and the human microbiome is made out of trillions of microscopic organisms (9, 25). Consuming fatty food, animal protein diet, alcohol and smoking routine, can alter the composition of

microorganisms in the gut (25). Comparable to other chronic diseases, dietary fat intake appears to be one of the most influential risk factors in progression of gut cancers (26).

Synthesis of bile acids. Bile acids are known as the end product of the utilized cholesterol, in fact, this synthesis is the major pathway of cholesterol catabolism in human (14). Even though different enzymes involved in bile acids synthesis are found in different types of cells, the liver considers as the only organ that allow the biosynthesis of this enzymes to completely occur (14). The initiation of the bile acid synthesis in the liver occurs by cholesterol 7α-hydroxylase (CYP7A1) enzyme, and the synthesis of the CA and the ratio of CA/CDCA are regulated by sterol 12α-hydroxylase (CYP8B1) enzyme (14, 15). Both (CYP8B1) and (CYP7A1) are regulated by bile acids via feedback repression mediated by farnesoid X receptor (FXR)-dependent induction of fibroblast growth factor 15/19 (FGF15/19) in the intestines (14, 15). FGF15/19 binds to FGF receptor 4/β-Klotho complex in hepatocytes which activates the JNK1/2 and ERK1/2 signaling cascade, down-regulates (CYP7A1) mRNA expression in the liver (15, 19). In the inner mitochondrial membrane, hydroxylation of cholesterol at the 27 position, acidic pathway initiated by mitochondrial sterol-27-hydroxylase (CYP27A1), in many tissues, (CYP27A1) is expressed extra-hepatically (14, 15). At the normal physiological condition, the acidic pathway is considered to be a minor pathway for bile acids synthesis and its accounts no more than 6%, however, in patients with cholesterol liver disease, the acidic pathway appears to be predominant pathway in synthesizing bile acids as (CYP7A1) is down regulated by the inflammation of small bowel in these patients (15, 19).

Humans, unlike rodents, are incapable to make 7-hydroxylating secondary bile acids back to the liver during the enterohepatic circulation, thus secondary bile acids in some humans can approach 60% of the biliary pool (15). Once the bile acids enter the terminal

ileum and proximal colon, instantly they are conjugated by prokaryotic enzymes known as bile salt hydrolase (BSH) (15). This enzyme, BSH, has different an affinity to taurine or glycine conjugates, BSH found in several bacterial divisions, such as 30% of Firmicutes, 14.4% of Bacteroides, and 8.9% of Actinobacteria (14). The detoxication function of BSH is reducing the bile salt level from the colon environment (14, 15).

Bile acid-dehydroxylating bacteria. Roughly, 400 to 800 mg per day of bile acids enter the intestinal, and most of this amount is transformed into different bile acids forms (12). Anaerobic gut bacteria are responsible for many transformations of bile acids within the human gut (27). *Clostridium scindens* has been reported to be capable of 7α-dehydroxylation. this activity converts CA and CDCA to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. In general, *Clostridium* species that produce 7α/β-dehydroxylation enzymes involved in bile acid metabolism include *C. scindens* and *C. hiranonis*, which belong to *Clostridium* cluster XIVa group, *C. sordelli* and *C. biofermentans*, which belong to *Clostridium* cluster XI group, and *C. leptum* which belongs to *Clostridium* cluster IV group (4, 28–30). Low dehydroxylation activity has been found in *C. hylemonae*, *C. sordelli*, *C. biofermentans*, and *C. leptum*; however, other *Clostridium* species are identified as highly active in bile acid dehydroxylation such as *C. scindens* and *C. hiranonis* (29, 30).

Genes involved in dehydroxylation. The following are known genes involved in the dehydroxylation of bile acids:

a. *baiA* gene: A 27-kDa polypeptide containing 249 amino acids is encoded by bai*A* gene and resembles the short-chain alcohol/polyol dehydrogenase gene family (31, 32). In *C. scindens*, different types of *baiA* genes have been found such as *baiA1* and *baiA*, which are identical, while *baiA2* is associated with the bai operon (31, 32).

***b. baiB* gene:** A 58-kDa polypeptide with 521 amino acids encodesa. CoA ligase, which depends on ATP, CoA, and Mg^{2+} and catalyzes the synthesis of CoA conjugated bile acids (31, 32).

***c. baiCD* gene:** A ~70-kDa polypeptide that encodes a steroid oxidoreductase enzyme, and this enzyme requires NAD^+ or $NADP^+$ for its activity (31, 32).

***d. baiH* gene:** A ~72 kDa-polypeptides with 661 amino acids is encoded by the *baiH* gene and is a steroid oxidoreductase. In *C. scindens*, the steroid oxidoreductase produced by *baiH* gene is specific for 3-dehydro-4-ursodeoxycholenoic acid and 3-dehydro-4-epicholenoic acid conjugated with CoA (31, 32).

***e. baiF* gene:** A 47.5-kDa polypeptide comprising 426 amino acids is encoded by the bai*F* gene (13). Bile acid CoA hydrolase activity has been found as a product from this gene (31, 32).

***f. baiG* gene:** A 50-kDa polypeptide containing 477 amino acids is encoded by this gene, and the transport of free primary bile acids, CA and CDCA, is facilitated by the bai*G* gene product; however, the secondary bile acids are not transported (31, 32).

***g. baiE* and *baiI* genes:** A ~19.5-kDa polypeptide holding 166 amino acids is encoded by bai*E* gene, and the enzyme encoded by this gene forms the stable intermediates 3-dehydro-4,6-deoxycholdienoic acid and 3-dehydro-4,6-lithocholdienoic acid, respectively. The *baiI* gene sequence shows homology with the *baiE* gene which indicates that *baiI* gene encodes the bile acid 7β-dehydratase (31, 32).

The removal of 7α-hydroxy group from primary bile acids requires multiple steps (Figure 2), and these steps include: ligation to CoA by *baiB* gene, oxidation of 3α-hydroxy by *baiA* gene, oxidation of C-4 and C-5 by *baiCD* gene, and 7α-hydroxy by *baiE* gene. All

of these reaction will be followed by consecutive reductions of NADP(H) at C-6- C-7, C4-C5, and 3-oxo followed by export of secondary Bile acids (15). Once bile acids enter the gut, metabolic activity will take place by variety of facultative and anaerobic bacteria, such as *Lactobacillus, Streptococcus, Staphylococcus, Escherichia, Enterococcus, Clostridium,* and *Bacteroides* (31).

Oxidation, reduction, and epimerization reactions. Microbial secretion of the hydroxysteroid dehydrogenase (HSDH) enzymes can oxidize and epimerize 3, 7, and 12-hydroxy groups of bile acids in the gastrointestinal tract (15, 33–35).

Epimerization of bile acids hydroxy group is a chemical conversion of one epimer to another in stereochemistry from α to β configuration with the generation of a stable oxo-bile acid intermediate. The epimerization reaction needs to occur, (6, 31, 36). This reaction happens as a microbial metabolic process on the steroid molecule (32). The intraspecies epimerization performed when one species metabolize either α-HSDH or β-HSDH of the hydroxyl group; however, the interspecies occurs when reaction happens on both α-HSDH and β-HSDH site by different species at the same time (31, 36). The reversible oxidation and reduction reactions depend on the redox of the environment, production of oxo-bile acids is more favorable at the higher redox potentials found on the mucosal surface, whereas the reduction of the oxo-bile acids might be more favorable under the low redox potentials in the large intestinal lumen (31). 3α /β HSDHs have been detected in most intestinal bacteria to catalyze the reversible oxidation/ reduction between 3-oxo- bile acids and 3α or 3β-hydroxy bile acids (34). Besides, 7α /β HSDHs are also common among the intestinal bacteria, however, the presence of the 7α /β-dehydrogenation is difficult to explain due to the irreversible of the 7α /β-dehydrogenation of bile acids (34).

In general, HSDHs have different reductive and oxidative pH optima, NAD(H) or NADP(H) requirements, molecular weight, and gene regulation (34).

12α-hydroxysteroid dehydrogenase HSDHs. HSDHs are found in a wide variety of microorganisms including bacteria and archaea, suggesting that steroid metabolism is an evolutionarily conserved mechanism that might serve different functions(12). There were only few studies reported that 12α-HSDH enzyme was actively found in *Clostridium* group P, strain C 48-50 ATCC 29733 (37). Although the 12α-hydroxy oxidation/reduction was observed in *Clostridium* group P, the epimerization to 12β-hydroxyl group was confirmed in *Clostridium paraputrificum* D 762-06 (38). 12α-HSDH was mainly found in *C. perfringens*, *C. leptum*, *Eubacterium lentum*, *Clostridium tertium*, *Clostridium difficile*, *C. paraputrificum* and crude preparation of *Pseudomonas* (31, 37, 38). Though bile acids generate up to 27 derivatives, up to date, it has not been reported that 12-oxolithocholic acid (12-oxoLCA) is able to be transformed by anaerobic gut bacteria to DCA (39). Consequently, it is significant to dedicate a study to understand the concept of potential transformation of 12-oxoLCA to DCA besides other oxo-bile acids derivatives by some of *Clostridium* spp. such as *C. scindens*, *C. hiranonis*, and *C. hylemonae*.

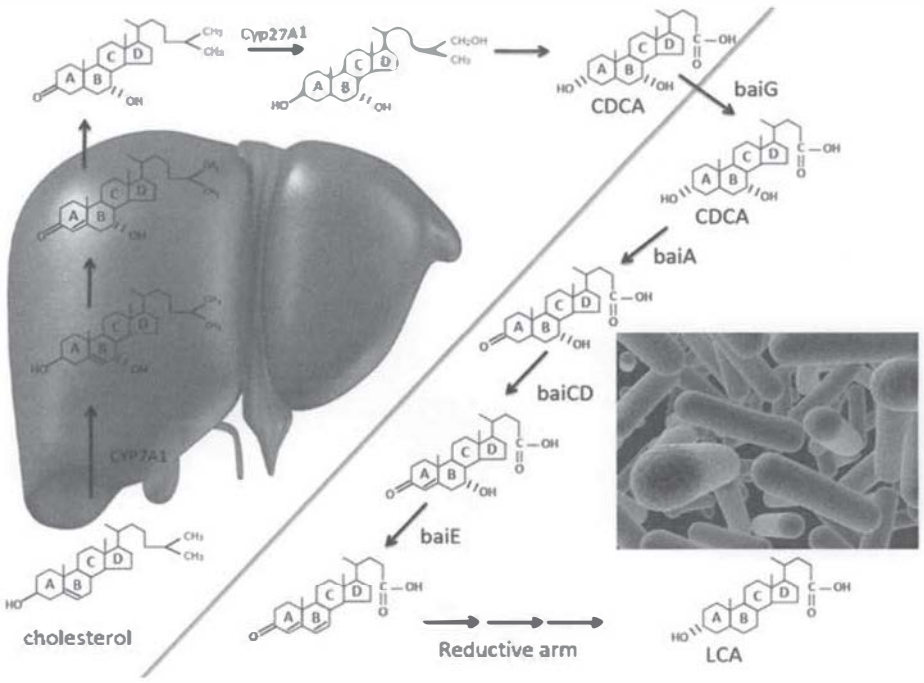


Fig 2. Biochemical pathway representing multiple steps involved in transforming of cholesterol to the primary bile acid chenodeoxycholic acid (CDCA) in the human liver, and the conversion of CDCA to lithocholic acid (LCA) in the human gut via anaerobic bacteria. Bacterial genes in the figure have been identified in *Clostridium scindens* ATCC 35704 (15).

2. OBJECTIVES

The objectives of this study were to:

- Determine the ability of *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 to reduce 12-oxolithocholic acid (12-oxoLCA) to deoxycholic acid (DCA) during growth (Fig 3).
- Identify the presence of a 12α-HSDH gene in *C. scindens* ATCC 35704.
- If present, express, purify, and characterize the 12α-HSDH gene product (protein) of *C. scindens* ATCC 35704.

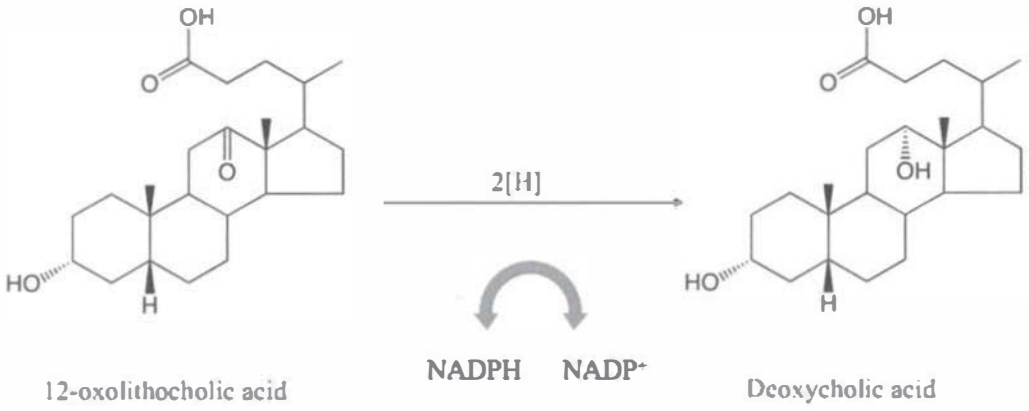


Figure 3. Conversion of 12oxolithocholic acid to deoxycholic acid by anaerobic gut bacteria.

3. MATERIALS AND METHODS

Bacterial strains. *Clostridium scindens* ATCC 35704, *Clostridium scindens* VPI 12708, *Clostridium hiranonis* DSM 13275, and *Clostridium hylemonae* DSM 15053 were obtained from Dr. Steven L. Daniel's culture collection at Eastern Illinois University and were revived from -80°C glycerol stocks.

Culture conditions and growth studies. Brain heart infusion (BHI) broth was the sole culture medium used for the cultivation of the bile acid-metabolizing bacteria used in the current study. BHI broth (200 ml) consisted of the following: 7.4 g brain heart infusion; 1.0 g yeast extract (Fisher Scientific); 0.4 g glucose (dextrose); 0.2 ml 0.1% resazurin; 1.5 g sodium bicarbonate. All components were added to a 500-ml Erlenmeyer flask containing a stir bar, thoroughly mixed, and brought to a boil (~15 min). After reaching a hard boil, the medium was gently bubbled with CO_2 until the medium color became brightly pink. Once the medium color reached a light pink in color, the medium was cooled in an ice bath while bubbling with CO_2. After the medium was cooled, 0.1 g of cysteine $\bullet HCl \bullet H_2O$ was added to the medium; bubbling was stoppered, and the headspace of the flask was flushed with CO_2. Once the medium became colorless, 10 ml was dispensed into 18 × 150-mm crimp-seal Bellco culture tubes (Bellco Glass, Inc., Vineland, NJ, USA) which were being flushed with CO_2. Tubes were stoppered and sealed with aluminum crimp seals and then autoclaved at 121°C for 15 min with fast exhaust. The pH of the medium after sterilizing and cooling was 6.6-6.8. In all experiments, sterile culture media were inoculated aseptically with 0.5 ml of inoculum using sterile 1.0-ml syringes and sterile 23-gauge needles. Inoculated tubes were incubated vertically in the dark at 37°C without shaking.

For growth studies done in the presence of individual bile acids (Table 1) or in the presence of a combination of two different bile acids (i.e., CA/DCA; CA/12oxoCDCA; and CA/12-oxoLCA), sterile bile acid stock solutions (10.6 mM per bile acid; see below for preparation and use) were added (0.1 ml) via sterile 1.0-ml syringes and sterile 23-gauge needles to tubes of sterile BHI broth (10 ml) prior to inoculation. Following inoculation, final concentration of a bile acid added to BHI broth was 0.1 mM.

For growth studies done in the presence of bile acids and potential regulatory substrates (Table 2), sterile substrate stock solutions (540 mM per substrate; see below for preparation and use) were added (0.2 ml) via sterile 1.0-ml syringes and sterile 23-gauge needles to tubes of sterile BHI broth (10 ml) prior to inoculation. Following inoculation, final concentration of a bile acid added to BHI broth was 10 mM.

Table 1. Preparation of bile acid solutions used in this study

Bile acid[a]	Molecular weight (g/mol)	Stock solution (g of bile acid per 10 ml of methanol; 10.6 mM)
CA	430.60	0.045644
3-oxoCA	406.56	0.043095
7-oxoDCA	406.56	0.043095
12-oxoCDCA	406.56	0.043095
DCA	414.6	0.043948
12-oxoLCA	390.56	0.041399
3-oxoDCA	390.56	0.041399
3, 12-dioxoLCA	388.54	0.041185
7, 12-dioxoLCA	404.54	0.042881
3, 7, 12-trioxoLCA	402.52	0.042667

[a] Bile acids used in this study: sodium cholate (CA); 3-oxocholic acid (3-oxoCA); 7-oxodeoxycholic acid (7-oxoDCA); 12-oxochenodeoxycholic acid (12-oxoCDCA); deoxycholate (DCA); 12-oxolithocholic acid (12-oxoLCA); 3-oxodeoxycholic acid (3-oxoDCA); 3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolithcholic acid (7, 12-dioxoLCA); and 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA).

Table 2. Molecular weight of different potential regulatory substrates used in this study

Substrate	Molecular weight (g/mol)	Stock solution (g of substrate per 100 ml of DRO water; 540 mM)
Sodium nitrate ($NaNO_3$)	84.99	4.58946
Pyruvic acid ($C_3H_4O_3$)	88.60	4.7844
Sodium formate ($NaHCO_2$)	68.01	3.67254
Sodium thiosulfate (NaS_2O_3)	248.18	13.40172
H_2	1.01	NA[a]

[a] NA, not applicable. 5 ml of H_2 gas was used per 10 ml of medium. Molar volume of a gas equals 1 mmole per 22.4 ml at STP; 5 ml of H_2 gas therefore equals approximately 0.22 mmoles per 10 ml or 22 mmoles per liter (22 mM).

Stock solutions. (i) Bile acids. A stock solution (10.6 mM) of each bile acid was prepared based on the following equation: molecular weight (g/mol) of bile acid × 0.0106 moles/liter × 0.01 liters = g of bile acid/10 ml of methanol (Table 1). Bile acid solutions were filtered using sterile 0.2-μm, 25-mm nylon syringe filters and sterile 10-ml syringes. Filtered methanolic solutions were aseptically transferred to sterile tubes, stoppered with sterile butyl rubber stoppers, crimp sealed and then made anoxic by flushing and pressurizing with argon gas. Anoxic stock solutions were stored at room temperature.

(ii) Potential regulatory substrates. Several substrates were examined their potential impact (i.e., regulation) on the reductive conversion of 12-oxoLCA to DCA. In other words, this experiment was designed to determine if sodium thiosulfate or sodium nitrate could serve as an alternative electron acceptor (i.e., reductant sink), thus decreasing 12-oxoLCA reduction to DCA. Furthermore, pyruvate, H_2 gas, and formate are known end products of glucose fermentation by *C. scindens* ATCC 35704 (reference needed) and were thus used to assess their ability to potentially regulate (e.g., via feedback inhibition) 12-oxoLCA transformation. All of the potential regulatory substrates were prepared as sterile, anoxic 540 mM stock solutions (Table 2); H_2 was tested by aseptically injecting 5 ml of gas into the headspace of tubes containing 10 ml of medium.

(iii) Mono-oxo bile acid TLC solvent system. The solvent system used to clearly visualize the mono-oxo bile acids (CA, 3-oxoCA, 7-oxoDCA, 12-oxoCDCA, DCA, 12-oxoLCA, and 3oxo-DCA) was cyclohexane, ethyl acetate, and glacial acetic acid at a ratio of 12:12:1 (v/v/v), respectively.

(iv) Di- and tri-oxo bile acid TLC solvent system. The solvent system used to for di- and tri-oxo bile acids (3, 12-dioxoLCA; 7, 12-dioxoLCA; and 3, 7, 12-trioxoLCA) was toluene, 1-4 dioxane, and glacial acetic acid at a ratio of 70:20:2 (v/v/v), respectively.

(v) Charring agent. Charring agent (500 ml) was used in TLC analysis for the visualization of bile acids on TLC plates and consisted of methanol (150 ml), water (150 ml, concentrated H_2SO_4 (10 ml), and $MnCl_2 \cdot 4H_2O$ (1 g). Charring agent solution was stored at room temperature.

(vi) Buffering agents. Streptavidin-binding buffer was prepared for use in the protein purification process and consisted of 2.42 g Tris base (20 mM), 8.78 g NaCl (150 mM), 100 ml of glycerol (10% glycerol), and 900 ml of distilled water. Elution buffer (QIAGEN) used in the affinity chromatography to wash the protein from the unbound proteins and release the desired protein from the ligand. For elution buffer, the recipe was 15 ml of streptavidin-binding buffer containing 0.00834 g d-desthiobiotin (2.5 mM). Other buffering agents used for pH optimization in the kinetics analysis of the recombinant enzyme included: citrate (pH 3.25 and 4), acetate (pH 4.5, 5.25, and 6), Tris-Cl (pH 8, 8.5, and 9), and glycine (pH 10 and 11). For enzymatic assays, all substrates such as CA; 3-oxoCA; 7-oxoDCA; 12-oxoCDCA; DCA; 12-oxoLCA; 3oxo-DCA; 3, 12-dioxoLCA; 7, 12-dioxoLCA; and 3, 7, 12-trioxoLCA were dissolved in methanol. All co-factors like $NADP^+$ and NADPH were dissolved in water, and 12α-HSDH enzyme was diluted in buffer with the correct pH for the direction of the reaction being tested. For Gel electrophoresis, 1X of electrophoresis running (TAE) buffer, consisting of 40 mM Tris, 20 mM acetate and 1mM EDTA in 1 liter of DI water, was used to run the gel electrophoresis to confirm the correctness of the DNA transformants.

Thin-layer chromatography (TLC). Thin-layer chromatography (TLC) (Whatman AL SIL G, 20 x 20 cm, 250 μm thick, non-UV) was used to detect bile acids (see Table 1) in standards (100 μM) prepared with 100% methanol, in standards (100 μM) prepared with

sterile BHI broth, and in cultures. The starting concentrations of all bile acids in cultures approximated 100 μM.

To detect bile acids in stationary-phase culture fluids, 1-ml samples of cultures were aseptically removed via sterile 1.0-ml syringes and sterile 23-gauge needles and transferred into two 2-ml microfuge tubes (0.5 ml of culture per microfuge tube). Each 0.5-ml sample was acidified by adding 100 μl of 3 N HCl and mixed by vortexing. To extract the bile acids from culture media, ethyl acetate (500 μl) was added to each acidified sample, and the microfuge tubes were capped, vortexed, and spun in a microcentrifuge for 1 min at 14,000 rpm. The upper organic phase which contained the extracted bile acids was carefully removed with a 1-ml Eppendorf pipettor and transferred to a labeled, small, screw-capped, wide-mouth vial. The 0.5-ml acidified samples were extracted a second time with ethyl acetate (500 μl), and the second extracts were transferred to the same vial that received the first extracts. The combined ethyl acetate extracts were dried at room temperature under a stream of nitrogen; once dried, methanol (100 μl) was added to the wide-mouth vial and then capped and mixed to resuspend the dried extracts. Resuspended extracts were used immediately for TLC analyses. The same procedure was used for bile acid standards prepared in sterile BHI broth. All manipulations were done in a fume hood and while wearing protective glasses, gloves, and a lab coat.

Resuspended extracts were spotted (50 μl) on a TLC plate at the designated spots; each spot was 3 cm from the bottom of the plate and 2.5 cm apart from one another and from the edge of the plate with a typical TLC plate allowing 7 samples to be analyzed at one time. With an Eppendorf pipettor and 100-μl tips, methanol-dissolved extracts were spotted onto TLC plates by applying a small amount of sample at a time, allowing that spot to dry, and then applying more of the sample in order to minimize creating a large sample

spot. In addition, to help quicken the spotting process and to reduce spot size, a hair dryer on low setting was used between applications in order to help accelerate methanol evaporation and sample drying. Theoretically, when initial bile acid concentrations were 100 μM in standards or in cultures, the amount of bile acid per spot on a TLC plate was equivalent to approximately 50 nmoles of bile acid per 50 μl of resuspended extract spotted.

Once all spots were dry, TLC plates were placed in a glass TLC chamber containing 50 ml of the solvent specific to the bile acid category (mono, di, or tri; see solvent compositions above). Run times for TLC plates approximated 3 h; afterwards, plates were removed, and the leading edge of the solvent front marked on the plate with a pencil. Plates were subsequently thoroughly dried at room temperature in the fume hood and then sprayed over the entire surface with the charring agent (water, methanol, concentrated H_2SO_4, and $MnCl_2$). Sprayed plates were dried in a small toaster oven for ~15 min at 150°C, observed under long wavelength UV light (365 nm) and photographed for future reference. Bile acids were identified by comparing R_f values of bile acids detected from cultures to R_f values of standard bile acids. R_f values represented the distance traveled in millimeters by the bile acid from the origin to the center of the bile acid spot divided by distance traveled in millimeters by solvent front from the origin to the leading edge of solvent front.

Gene Cloning. (i) Polymerase chain reaction and DNA amplification. PCR was used to provide sufficient 12α-HSDH gene-containing segments of DNA from *C. scindens* ATCC 35704 for gene cloning. Genomic DNA was extracted for molecular cloning using the Fast DNA isolation kit from Mo-Bio (Carlsbad, CA, USA) as described in the manufacturer's protocol. The 12α-HSDH gene was amplified using Phusion High Fidelity Polymerase (Stratagene La Jolla, CA) as described by the manufacturer's protocol, and annealing time for the amplification was determined to be 63.3°C for *C. scindens*. PCR

reactions conditions are described in Table 3. Primers used in this study were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

(ii) Purification of the insert. PCR product was cleaned using a PCR purification Kit (Qiagen, Valencia, CA, USA), according to the manufacturer protocol. PB buffer (5X volume) was added to each PCR reaction (250 µl of PB buffer to 50 µl of reaction volume). The mixture was transferred to a spin column and centrifuged for 30 sec at 13,000 rpm. Flow through was discarded, and 750 µl of PB buffer was added and recentrifuged for 30 sec at 13,000 rpm. Flow through was again discarded and the column recentrifuged for another 30 sec at 13,000 rpm to remove any extra fluid. Next, the column was placed in a microfuge tube, and 50 µl of H_2O was added to the column. After 3 min, the column was centrifuged for 1 min at 13,000 rpm and the flow through collected. The concentration of DNA in the collected flow through was measured using Nanodrop (Fisher Scientific™).

Table 3. Amplification of 12α-HSDH gene	
Component name (concentration in stock solution)	Amount added
Molecular grade H_2O	35.5 µl
5X Buffer	10 µl
10 mM dNTP (10 mM)	1 µl
Template DNA (*C. scindens* genome)	1 µl
FWD primer (5'-forward primer-3') (10 µM) ATATATGGTACCGATGGGTATATTTGACGGAAAAACAGCTA	1 µl
REV primer (5'-reverse primer-3') (10 µM) ATATATAAGCTTTTATGGGCGCTGTCCCATGC	1 µl
Phusion polymerase (20 units/ml)	0.5 µl
Total volume	50 µl

(iii) Recombinant DNA. PCR product and the pET51b vector were double digested in order to insert the amplified DNA fragment of 12α-HSDH into the pET51b vector. The pET51b vector was obtained from Novagen (San Diego, CA, USA). pET51b vector was digested with the appropriate restriction endonucleases and treated with DNA ligase to allow a successful insertion of the target DNA 12α-HSDH. The double digestion run on the vector was applied by following the same PCR process explained by the manufacturer's protocol using the Phusion High Fidelity Polymerase (Stratagene La Jolla, CA). Restriction enzymes for *C. scindens* were HindIII and BamHI, and the buffer used was Buffer 3.1. All restriction enzymes were obtained from NEB (Ipswich, MA).

(iv) Gel electrophoresis. Gel electrophoresis was applied to confirm the plasmid transformants. In 125 ml flask, 50 ml of 1X TEA buffer was added to 500 mg of electrophoresis grade agarose, mixed then placed in a microwave for 30-45 sec. After the liquid had boiled, 5 µl of ethidium bromide was carefully added and mixed until dissolved. The appropriate size comb was placed into the gel tray, and the gel was poured into the tray gently and kept at room temperature for 20 min to solidify. Once solidified, the gel was placed into the gel electrophoresis apparatus, and TAE buffer (1X) was added until it was about a half inch above the gel. Gels were loaded by placing 6 µl of DNA ladder in first well while the other wells were loaded by first mixing 1 µl of loading dye with 5 µl of PCR reaction product and then transferring the entire amount to a well, loading dye and DNA ladder were obtained from Thermo Fisher Scientific. Gels were run for 1 h at 120 V, and results were visualized by Gel Doc.

(v) Transformation of DH5α cells. The recombinant plasmids (12α-HSDH gene cloned into the pET-51b (+) vector) were transformed into *Escherichia coli* DH5α cells. DH5α cells were revived from glycerol stocks stored at -80°C. In microfuge tube, 2 µl of ligation product, (plasmid contains the gene of interest), was added to 25 µl of DH5α competent cells. Tubes containing competent cells with the ligated products were cooled in ice for 30 min then placed in the thermomixer for 40 sec at 43°C, then iced again for 5 min. Aseptically, 200 µl of super optimal broth with glucose (SOC) medium was added to the mixture, tubes were placed in the shaker ~150 rpm for 1 h at 37°C. Each sample was transferred to Lysogeny Broth (LB) agar plate supplemented with ampicillin (100 µg/ml), and cells were spread over the agar surface and incubated overnight at 37°C. From each transformation, a single colony was picked with an Eppendorf tip, put into small conical tube containing 5 ml of LB broth supplemented with ampicillin (100 µg/ml) and incubated

overnight with shaking (~250 rpm) at 37°C. Cells in cultures were harvested by pelleting at 10,000 rpm for 3 min; cell pellets of DH5α were stored at -80°C for plasmid extraction process.

LB broth was the culture medium used for the growth of *Escherichia coli* DH5α cells and the overexpression of the recombinant plasmid. LB broth consisted of the following per liter: 10 g tryptophan, 5 g yeast extract, 5 g NaCl, and 15 g of agar when needed. All components were added to 2-l flask, covered with aluminum foil, and then autoclaved at liquid cycle for 1 h at 121°C. The medium was cooled to room temperature then aseptically supplemented with ampicillin (100 μg/ml of medium) and chloramphenicol (50 μg/ml of medium) as indicated.

(vi) Plasmid extraction/purification and gel electrophoresis. The recombinant plasmid was extracted and purified according to the manufacturer protocol QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Gel electrophoresis (see protocol above) was applied to confirm the plasmid transformants.

(vii) Gene sequencing. Sanger DNA sequencing was then used to determine the proper insertion of the gene into the plasmid and to verify the 12α-HSDH gene sequence. Sequencing and data analysis were done at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign.

Overexpression of the Recombinant Protein. (i) Transformation into BL-21 CodonPlus. The extracted recombinant plasmids containing the cloned gene were transformed into *E. coli* BL-21CodonPlus (DE3) RIPL chemically competent cells by using heat shock method. BL-21 cells were revived from glycerol stocks stored at -80°C at University of Illinois at Urbana-Champaign.

In microfuge tube, 1µl of recombinant plasmid-containing solution was added to 30-50 µl of BL-21 competent cells. Tubes containing competent cells and recombinant plasmid-containing solution were cooled in ice for 30 min then placed in the thermomixer for 40 seconds at 43°C, then iced again for 5 min. Aseptically, 200 µl of super optimal broth with glucose medium was added to the mixture, and tubes were placed on a shaker (~150 rpm) for 1 h at 37°C. Cells were grown on LB plates supplemented with chloramphenicol (50 µg/ml) and ampicillin (100 µg/ml) and incubated overnight at 37°C.

(ii) Overexpression. Colonies were picked using sterile pipette tips and transferred to 10 ml of LB broth supplemented with chloramphenicol (50 µg/ml) and ampicillin (100 µg/ml). Culture tubes were incubated for 6 h on a shaker (~250 rpm) at 37°C. The entire volume of a 10-ml culture was transferred to a flask containing one liter of LB broth supplemented with chloramphenicol (50 µg/ml) and ampicillin (100 µg/ml). Flasks were incubated at 37°C on a shaker (~220 rpm) until growth (OD_{600nm}) reached approximately 0.3 (about 3 h). Recombinant protein production was then induced by adding 750 µl of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the LB cultures; flasks were incubated overnight at 16°C. Following overnight incubation, cells were pelleted in a preparative centrifuge for 30 min at 5,000 rpm and 4°C. Pelleted cells were then re-suspended in 30 ml of cold binding buffer (pH 7.9) containing 20 mM Tris HCl, 150 mM NaCl, 10% glycerol, and 10 mM 2-mercaptoethanol.

Enzyme Purification. (i) Cell lysis. Cells that induced with IPTG were treated by adding 25 µl of beta-mercaptoethanol B-ME and 25 µl of DNase were added along with 750 µl of lysozyme to the same mixture, then tubes were vortexed to mix. After that, cell suspension was passed several times through an EmulsiFlex C-3 cell homogenizer (Avestin, Ottawa, Canada) according to manufacturer's protocol. Soluble crude lysate was

separated from insoluble crude lysate by centrifugation at 13,000 rpm for 30 min at 4°C. Supernatant fluid (50 µl of soluble crude lysate) and cell pellet (50 µl of insoluble crude lysate) was saved for SDS-PAGE analysis.

(ii) Purification via affinity chromatography. The recombinant 12α-HSDH was purified using Strep-Tactin® resin according to the manufacturing protocol (IBA GmbH).

Enzyme Characterization. (i) Kinetic analysis of recombinant 12α-HSDH. Enzyme reactions were determined by observing the oxidation and reduction activities in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^+$) and the reduced form (NADPH) at 340 nm for 1.5 min; reactions were observed with the recombinant enzyme 12α-HSDH from *C. scindens* ATCC 35704 and various bile acid substrates. Data was analyzed using Michaelis-Menten equation and a nonlinear regression method using enzyme kinetics methods in GraphPad Prism (GraphPad software, La Jolla, CA, USA). 12α-HSDH activities were also analyzed by (i) varying recombinant enzyme concentrations for the oxidative direction and reductive directions; (ii) varying substrate concentrations for DCA and 12-oxo-LCA; and varying $NADP^+$ (DCA) and NADPH (12-oxoLCA).

(ii) The pH optimization of recombinant 12α-HSDH. Optimal pH was determined by varying the pH level of the assay in order to reach the optima enzyme activity in both the oxidative direction (DCA/NADP+) and the reductive direction (12-oxoLCA/NADPH).

(iii) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The purity of the recombinant protein was determined by SDS-PAGE analysis. 2 µl of the extracted protein was loaded into each compartment in the prepared gel, and the protein bands were visualized by Coomassie brilliant blue G-250 staining. Protein concentrations

were measured based on the portion extinction coefficient and molecular mass at the University of Illinois at Urbana-Champaign.

4. RESULTS

Growth. *C. scindens* ATCC 35704, *C. scindens* VPI 12708. *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 were grown in BHI broth to provide the nutrients necessary for the organisms to reach optimum growth during this study. When methanolic solutions of bile acids were added to BHI broth (final concentrations were 0.1 mM for bile acids and 0.9% for methanol in culture medium after inoculation), growth levels and patterns were essentially unchanged by these organisms, indicating that at the concentrations tested 12-oxoLCA did not appear to positively or negatively impact the growth profiles of these organisms (Figure 4-7). *C. scindens* yielded the greatest growth compared to *C. hylemonae* and *C. hiranonis*, respectively (Figure 4-7). All tested organisms reached a stationary phase in 24-h in absence of bile acids. However, *C. hylemonae* and *C. hiranonis* took 72-h to reach the stationary phase, while *C. scindens* reached the maximum growth in 48-h in the presence of bile acids substrate.

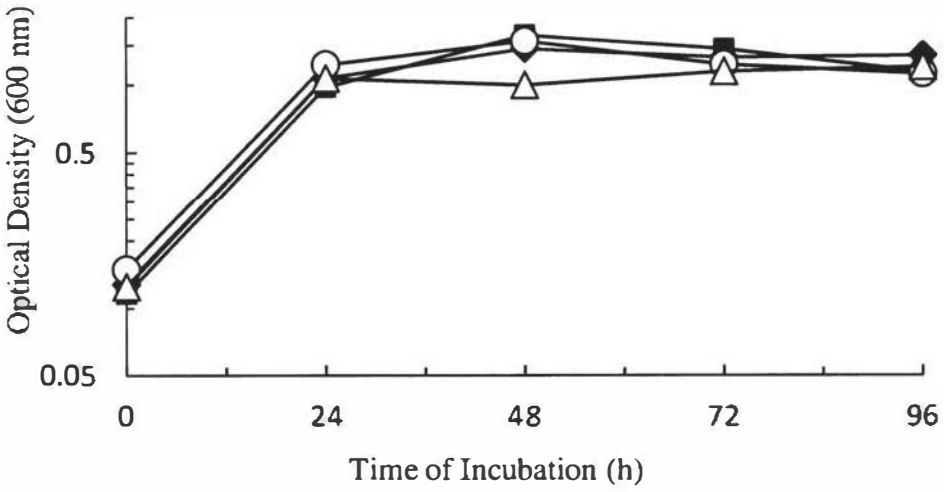


Fig 4. Comparison of the growth of *Clostridium scindens* ATCC 35704 in brain heart infusion (BHI) broth and BHI broth supplemented with 0.1 mM bile acid. Values represent the means of duplicate tubes. Symbols: ◆, BHI alone; ■, cholic acid; ○, deoxycholic acid; △, and 12-oxolithicholic acid.

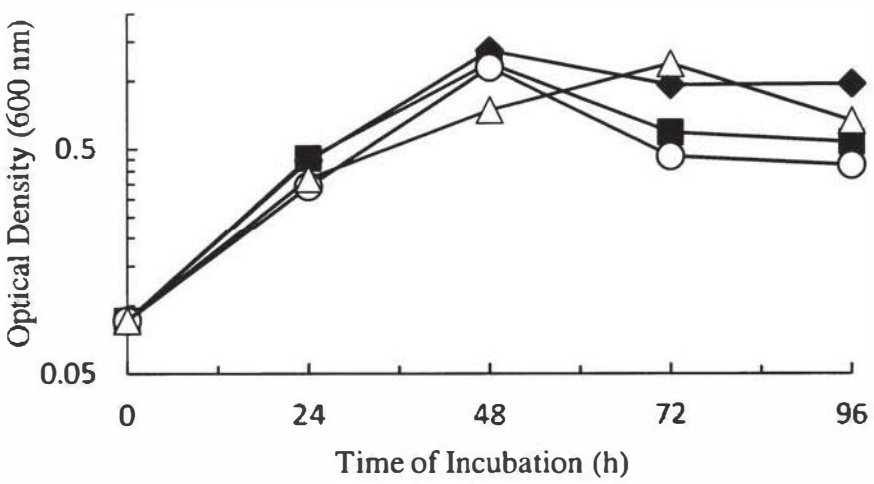


Fig 5. Comparison of the growth of *Clostridium scindens* VPI 112708 in brain heart infusion (BHI) broth and BHI broth supplemented with 0.1 mM bile acid. Values represent the means of duplicate tubes. Symbols: ◆, BHI alone; ■, cholic acid; ○, deoxycholic acid; △, and 12-oxolithicholic acid.

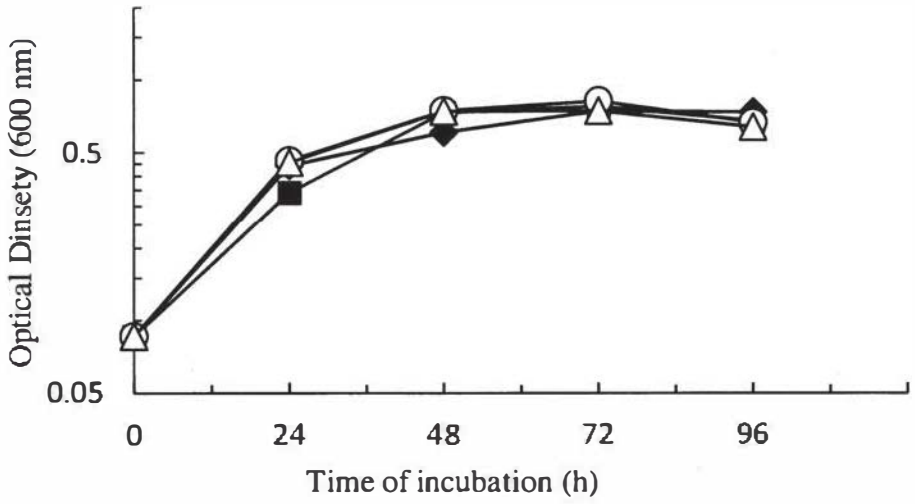


Fig 6. Comparison of the growth of *Clostridium hiranonis* DSM 13275 in brain heart infusion (BHI) broth and BHI broth supplemented with 0.1 mM bile acid. Values represent the means of duplicate tubes. Symbols: ◆, BHI alone; ■, cholic acid; ○, deoxycholic acid; △, and 12-oxolithicholic acid.

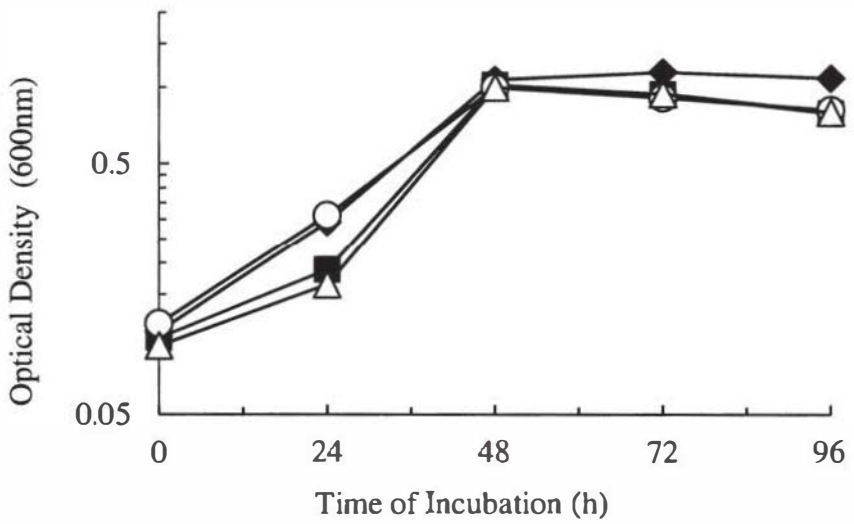


Fig 7. Comparison of the growth of *Clostridium hylemonae* DSM 15053 in brain heart infusion (BHI) broth and BHI broth supplemented with 0.1 mM bile acid. Values represent the means of duplicate tubes. Symbols: ◆, BHI alone; ■, cholic acid; ○, deoxycholic acid; △, and 12-oxolithicholic acid.

Conversion of 12-oxoLCA to DCA. *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275 and *C. hylemonae* DSM 15053 were all capable of converting 12-oxoLCA to DCA when grown in BHI broth supplemented with 100 μM 12-oxoLCA (Figure 8). It should be noted that 12-oxoLCA and DCA were never observed in BHI cultures in the absence of 12-oxoLCA supplementation (data not shown). A potential reaction for the formation of DCA from 12-oxoLCA is shown in Figure 8 and represents a reduction which consumes 2 reducing equivalents (2H) during this conversion

As a novel investigation, it was important to validate that the conversion of 12-oxoLCA to DCA was the result of enzymatic activity (abiotic activity) by the organisms being tested rather than by chemical/physical activity (abiotic activity). In this regard, when 12-oxoLCA was incubated anaerobically at 37°C in sterile BHI broth (without inoculation) for five consecutive days, TLC analysis did not show any detectable conversion of 12-oxoLCA to DCA or any other products (Figure 9-10). 12-oxoLCA dissolved in methanol during preparation of standards was not abiotically converted to DCA. In addition, spent culture fluids were also examined to confirm that 12-oxoLCA reduction to DCA was not the result of dead cells, cell debris, or metabolic end products from these organisms (Figure 11). As a result, in the absence of actively growing cells, 12-oxoLCA was not reduced to DCA, indicating that the growth of these organisms (abiotic activity) was responsible for the observed reduction (Figure 10-11).

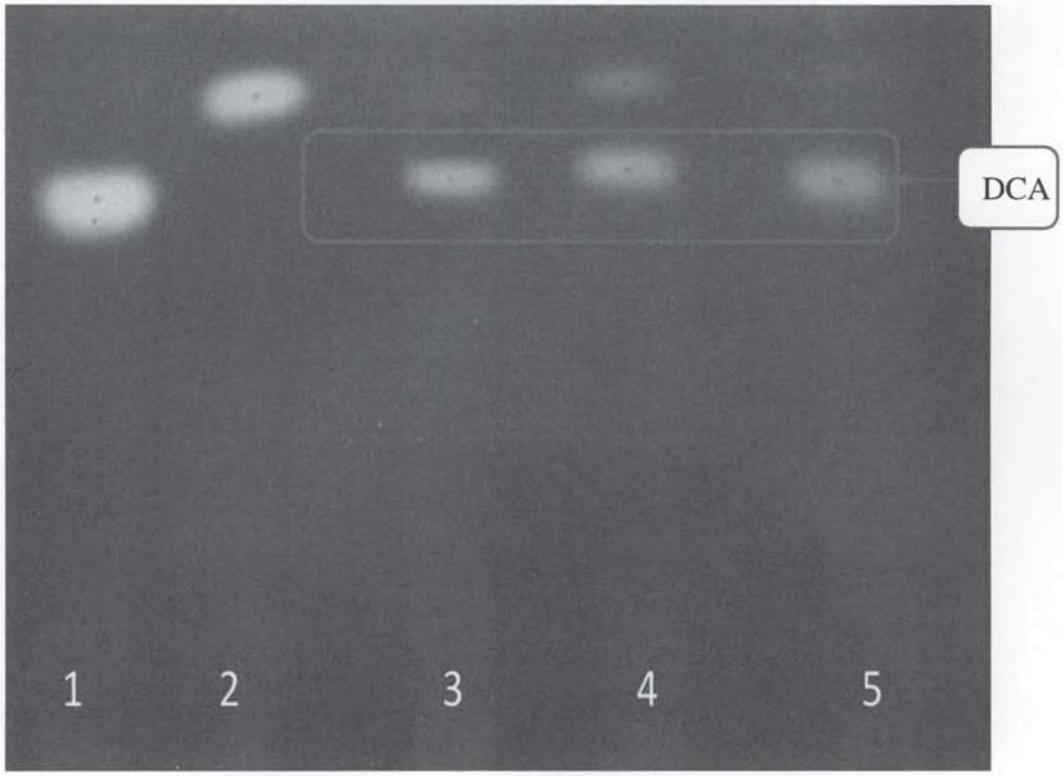


Fig 8. Metabolism of 12-oxolithocholic acid (12-oxoLCA) by *Clostridium scindens* ATCC 35704, *Clostridium hylemonae* DSM 15053, and *Clostridium hiranonis* DSM 13275. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lane: [1], 100 μM of methanolic standard of deoxycholic acid (DCA); [2], 100 μM of methanolic standard of 12-oxoLCA; [3], *C. scindens* with 12-oxoLCA; [4], *C. hylemonae* with 12-oxoLCA; and [5], *C. hiranonis* with 12-oxoLCA.

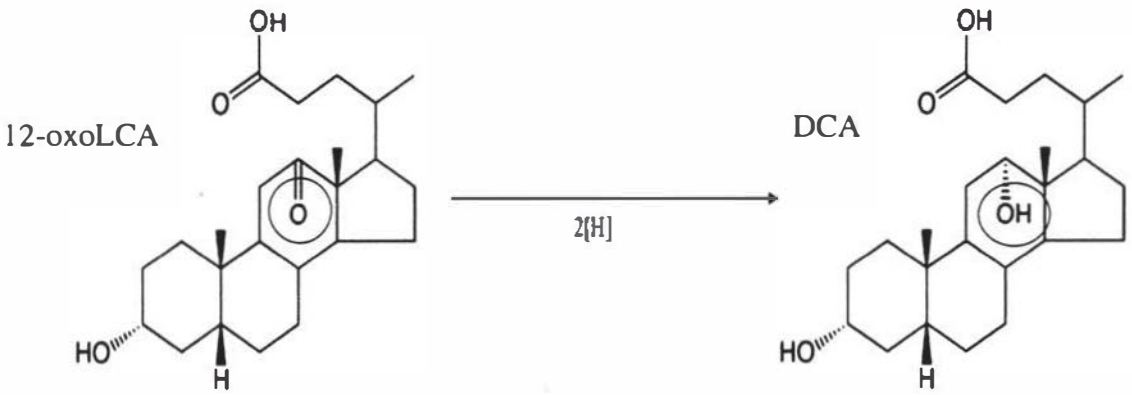


Fig 9. Chemical reaction for the conversion (reduction) of 12-oxolithocholic acid (12-oxoLCA) to deoxycholic acid (DCA).

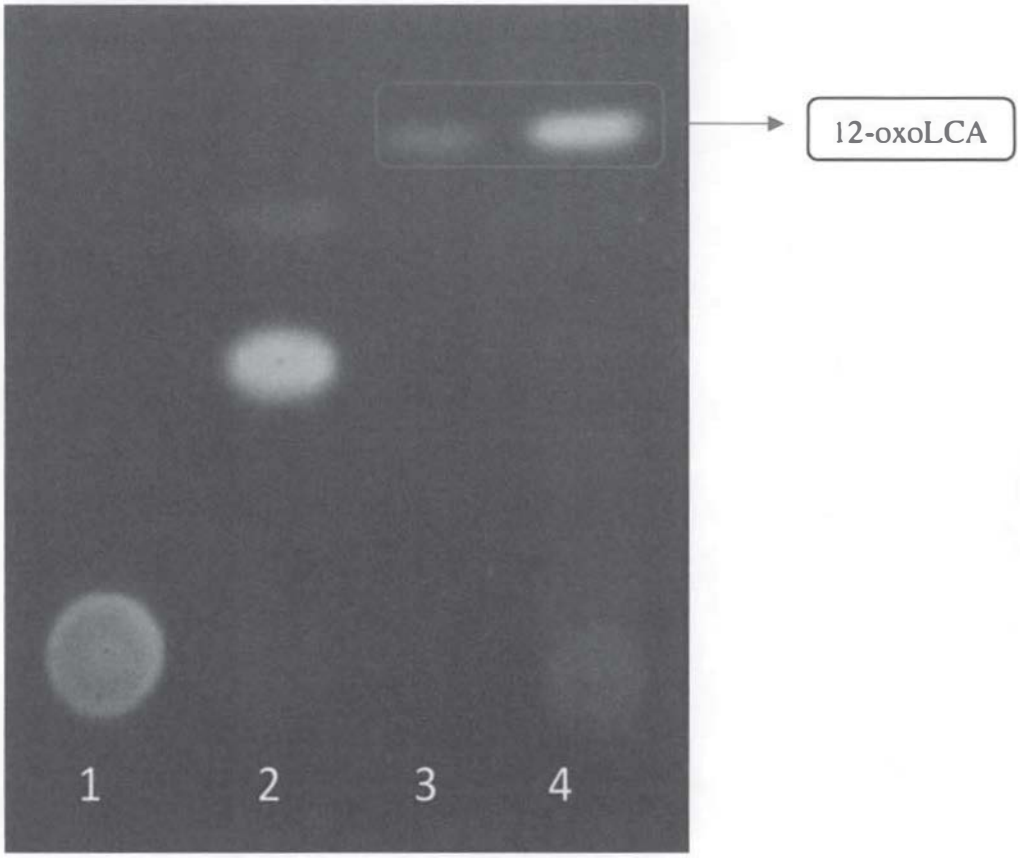


Fig 10. Potential impact of brain heart infusion broth (BHI) and methanol on stability of 12-oxolithocholic acid (12-oxoLCA). 12-oxoLCA (100 μM) was incubated in BHI broth for five days at 37°C. Bile acids were extracted, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lane: [1], 100 μM of methanolic standard of cholic acid (CA); [2], 100 μM of methanolic standard of deoxycholic acid (DCA); [3], 100 μM of methanolic standard of 12-oxoLCA; and [4], 12-oxoLCA incubated for five days in sterile BHI.

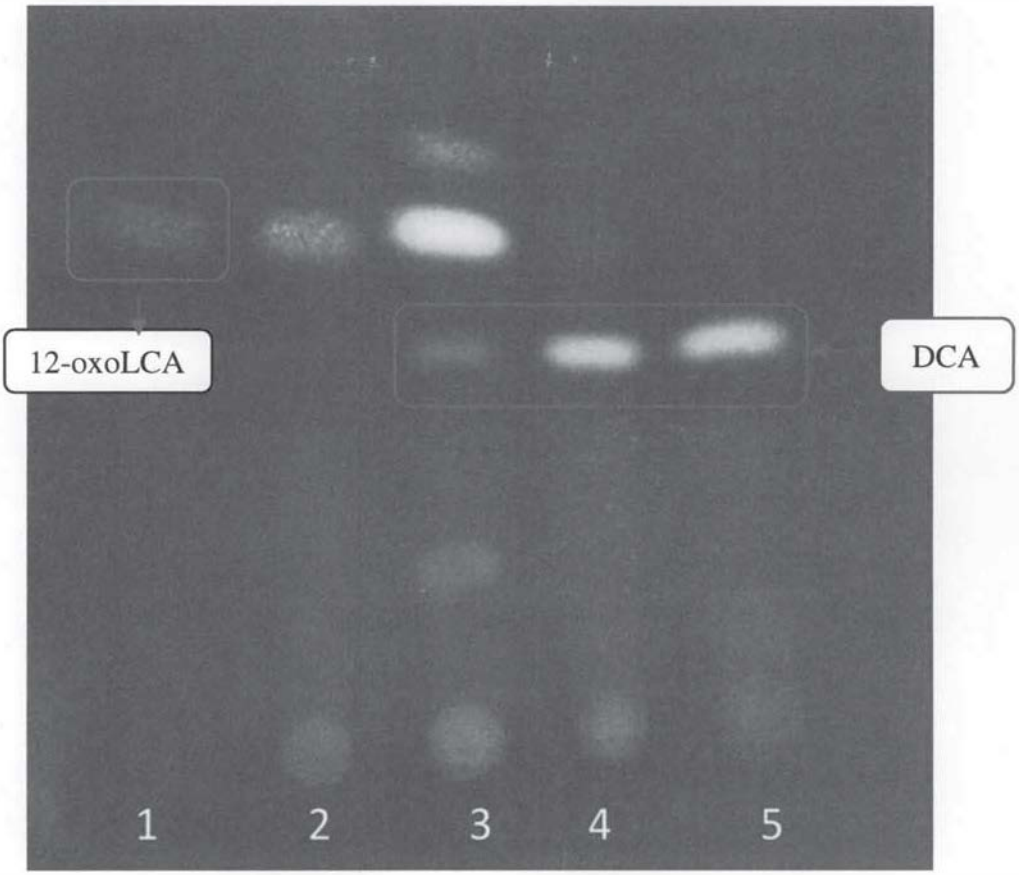


Fig 11. Metabolism of 12-oxolithocholic acid (12-oxoLCA) and deoxycholic acid (DCA) by *Clostridium scindens* ATCC 35704. As indicated otherwise, organism was grown in brain heart infusion broth (BHI) supplemented with 100 µM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], spent BHI broth culture incubated with 100 µM 12-oxoLCA; [2], 100 µM methanolic standard of 12-oxo-LCA; [3], a methanolic standard mix containing 100 µM of 12-oxoLCA and 100 µM of DCA; [4], *C. scindens* with 12-oxoLCA; and [5], *C. scindens* with DCA.

7α-dehydroxylation. *C. scindens* ATCC 35704 and *C. hiranonis* DSM 13275. actively dehydroxylated CA to DCA when CA was added to BHI broth. In contrast, *C. hylemonae* DSM 15053 showed a reduced capacity for generating DCA from CA (Figure 12). However, activity levels were different (increased) when *C. hylemonae* DSM 15053 cells were induced by sequential passages in BHI supplemented with CA followed by TLC analysis (Figure 13).

Specificity of bile acid transformation. (i) mono-oxo bile acids. *C. scindens* VPI 12708 was apparently unable to transform 12-oxoLCA to DCA (Figure 14; Table 5), however, *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 were clearly able to convert 12-oxoLCA to DCA by reducing the hydroxyl group at carbon atom position 12 to an oxo group (Figure 9 and 14). In contrast, only *C. scindens* ATCC and *C. hiranonis* DSM 13275 were able to transform 7-oxoDCA to DCA (Figure 15-16; Table 4), and *C. scindens* ATCC 35704 generated CA from 7-oxoDCA (Figure 15; Table 4). Furthermore, *C. scindens* ATCC 35704, were unable to metabolize either DCA (Figure 16; Table 4) or 3-oxoDCA during growth (Figure 17-19; Table 4-5). Unexpectedly, instead of generating DCA, *C. scindens* ATCC 35704 and *C. hylemonae* DSM 15053 transformed 12-oxoCDCA completely to 7-oxoDCA (Figure 18- 19; Table 4-5), while *C. hiranonis* DSM 13275 partially metabolized 12-oxoCDCA to 7-oxoDCA (Figure 20; Table 4). *C. scindens* ATCC 35704 and *C. hylemonae* DSM 15053 transformed 3-oxoCA incompletely to CA, and some of the 3-oxoCA was observed as a non-metabolized substrate (Figure 18-19; Table 4). Moreover, only *C. hiranonis* DSM 13275 was able to generate DCA and CA from 3-oxoCA (Figure 20; Table 4- 5).

(ii) Di- and tri-oxo bile acids. Di- and tri-oxo bile acids were also tested to determine the possible capability of these organisms to metabolize other oxo-derivatives. *C. scindens*

ATCC 35704 transformed the 7, 12-dioxoLCA partially to both DCA and CA (Figure 21; Table 4- 5). Yet, C. *hylemonae* DSM 15053 did not yield any DCA from the 7, 12-dioxoLCA; instead, this organism removed the 7-oxo and 12-oxo groups from the substrate and reduced the 3-hydroxyl group to produce 3-oxoCA as a final end product (Figure 22; Table 4- 5). 3, 7, 12-oxoLCA was the only tri-oxo bile acid evaluated in the present study (Table 5), and this oxo-substrate was transformed to DCA only by C. *hylemonae* DSM 15053 (Figure 23; Table 4). However, 7, 12-dioxoLCA was entirely transformed to DCA by *C. hiranonis* DSM 13275 (Figure 24; Table 4); Nevertheless, *C. scindens* ATCC 35704 and *C. hiranonis* DSM 13275 generated CA by reducing hydroxyl groups at the 3, 7, and 12 positions of tri-oxoLCA (Figure 24-25; Table 4). Additionally, *C. scindens* ATCC 35704 and *C. hiranonis* DSM 13275 also metabolized tri-oxoLCA differently by reducing the hydroxyl groups at only the 7 and 12 positions and ending up with 3-oxoCA (Figure 24; Table 4). The second di-oxo bile acid was tested in this study was 3, 12-oxoLCA (Table 5), and *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 generated 3-oxoDCA from 3, 12-oxoLCA (Figure 26-28; Table 4). This biotransformation appears to occur when the 12-oxo group is reduced to a 12-hydroxyl group, yielding 3-oxoDCA. The specific reactions and enzymes used by *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 to metabolize these different oxo-derivatives are presently unknown. Further studies will be required to resolve these reactions, including studies designed to reveal the conditions which might influence oxo-bile acid metabolism by these organisms (Table 4).

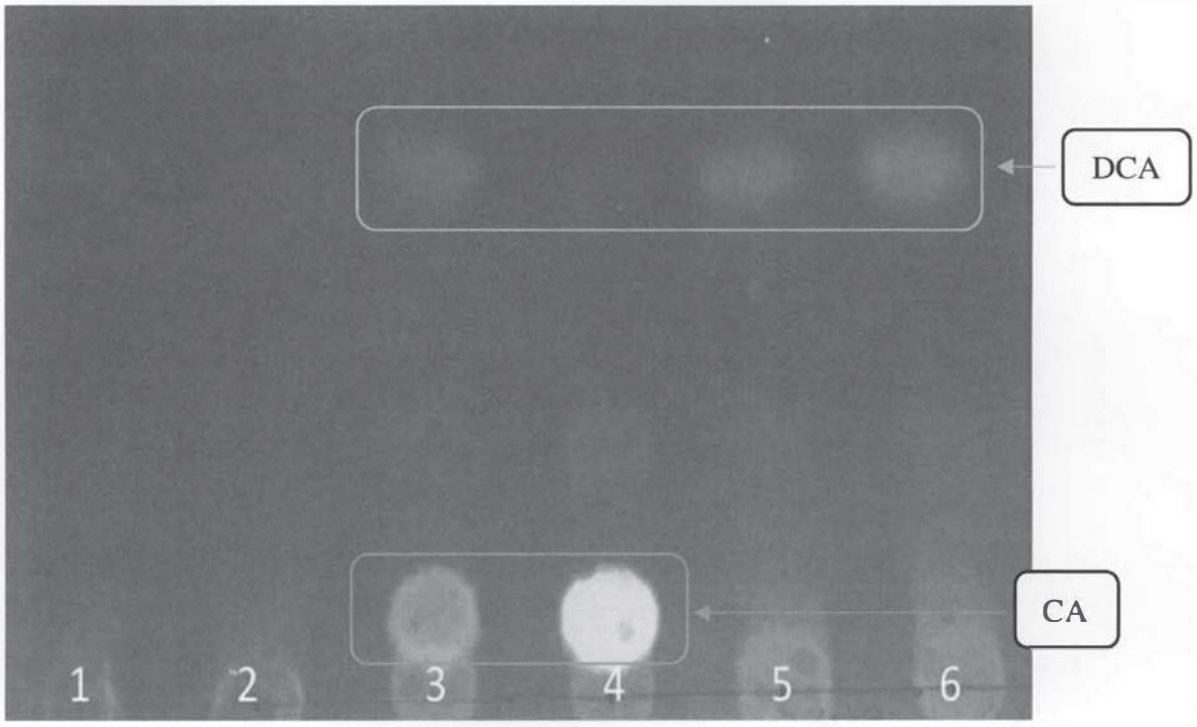


Fig 12. Metabolism of cholic acid (CA) and 12-oxolithocholic acid (12-oxoLCA) by *Clostridium scindens* ATCC 35704 and *Clostridium hylemonae* DSM 15053. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], methanol with *C. scindens*; [2], methanol with *C. hylemonae*; [3], CA with *C. scindens*; [4], CA with *C. hylemonae;* [5], 12-oxoLCA with *C. scindens*; and [6], 12-oxoLCA with *C. hylemonae.*

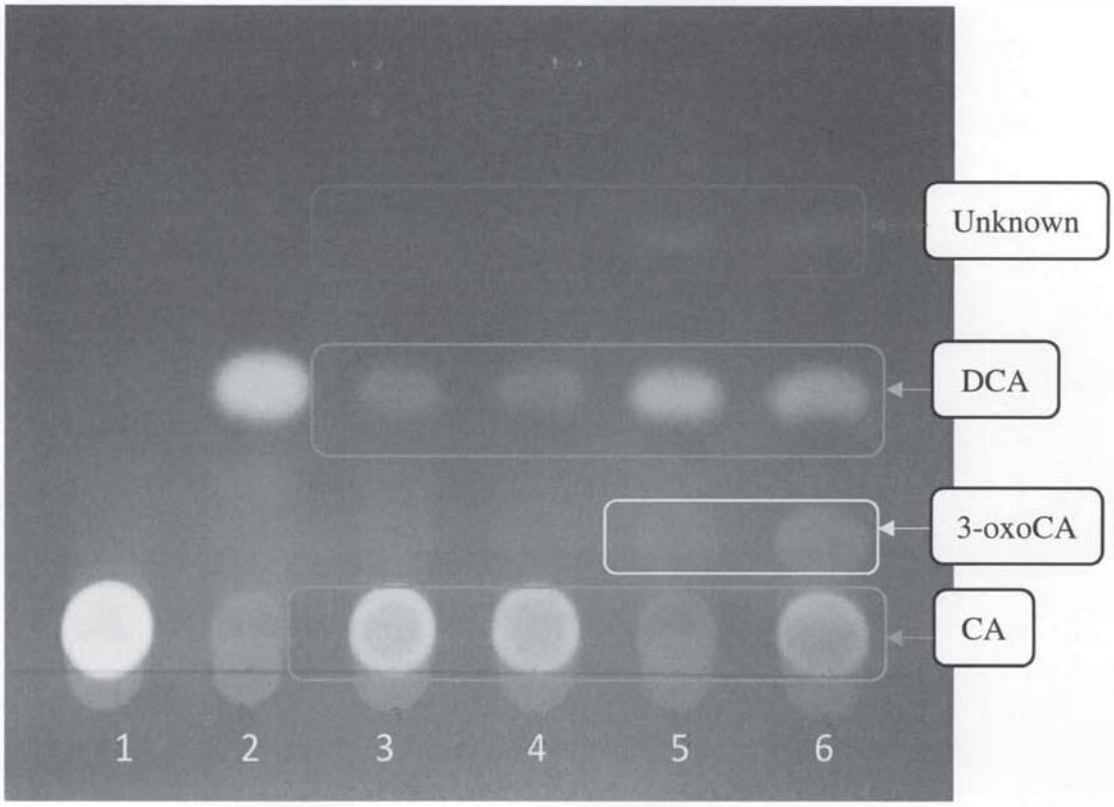


Fig 13. Metabolism of cholic acid (CA) by *Clostridium hylemonae* DSM 15053. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM CA, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 50 μM methanolic standard CA; [2], standard deoxycholic acid (DCA); [3, 4], 1st passage of *C. hylemonae* with CA; [5, 6], 2nd passage of *C. hylemonae* with CA.

Table 4. Bile acids transformations by *Clostridium scindens* ATCC 35704, *Clostridium hiranonis* DSM 13275, and *Clostridium hylemonae* DSM 15053.

Bile acid added to culture [a]	R_f value	Bile acids detected after growth (24-72 h)					
		CA	3-oxoCA	7-oxoDCA	12-oxoCDCA	DCA	3-oxoDCA
CA	0.04 ± 0.02 [b]	*C. hyle* [d]	ND [e]	ND	ND	*C. sci* *C. hir*	*C. hir*
3-oxoCA	0.21 ± 0.03	*C. sci* *C. hyle* *C. hir*	*C. sci* *C. hyle*	*C. hir*	ND	*C. hir*	*C. hir*
7-oxoDCA	0.14± 0.03	*C. sci*	ND	*C. hyle* *C. hir*	ND	*C. sci* *C. hir*	*C. hir*
12-oxoCDCA	0.27 ± 0.06	ND	ND	*C. sci* *C. hyle* *C. hir*	*C.hir*	ND	ND
DCA	0.38 ± 0.14	ND	ND	ND	ND	*C. sci* *C. hyle* *C. hir*	ND
3-oxoDCA	0.56 ± 0.11	ND	ND	ND	ND	ND	*C. sci* *C. hyle* *C. hir*
12-oxoLCA	0.51 ± 0.14	ND	ND	ND	ND	*C. sci* *C. hyle* *C. hir*	ND
3,12-dioxo-LCA	0.55 ± 0.01[c]	ND	ND	ND	ND	ND	*C. sci* *C. hyle* *C. hir*
7,12-dioxo-LCA	0.22 ± 0.02[c]	*C. sci*	*C. hyle*	ND	ND	*C. sci* *C. hir*	ND
3,7,12-trioxo-LCA	0.46 ± 0.03[c]	*C. sci* *C. hir*	*C. sci* *C. hir*	ND	ND	*C. hyle*	ND

[a] Final concentration of bile acid added to BHI culture was 0.1 mM.

[b] TLC solvent used to detect mono-oxobile acids: (cyclohexane, ethyl acetate, and glacial acetic acid; 12:12:1).

[c] TLC solvent used to detect di and tri-oxobile acids; (toluene, 1- 4 dioxane, and glacial acetic acid; 70:20:2).

[d] Detection limit of bile acid was determined at ≤ 10 µM.

[e] Not detected.

Abbreviation: (CA), cholic acid; (3-oxoCA), 3-oxocholic acid; (7-oxoDCA), 7-oxodeoxycholic acid; (12-oxoCDCA), 12-oxochenodeoxycholic acid; (DCA), deoxycholic acid; 3-oxoDCA, 3-oxodeoxycholic acid; (12-oxoLCA), 12-oxolithocholic acid; (3, 12-dioxoLCA), 3, 12-dioxolithocholic acid; (7, 12-dioxoLCA), 7, 12-dioxolithocholic acid; (3, 7, 12-trioxoLCA), 3, 7, 12-trioxolithocholic acid. *C. sci, Clostridium scindens* ATCC 35704; *C. hir. Clostridium hiranonis* DSM 13275; and *C. hyle, Clostridium hylemonae* DSM 15053.

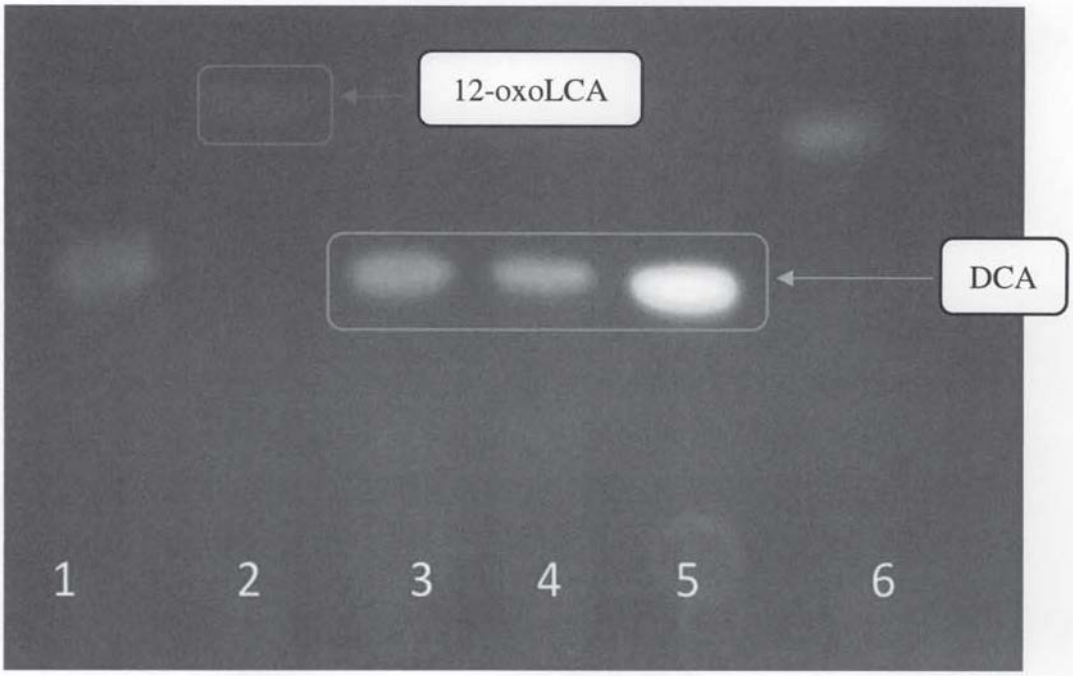
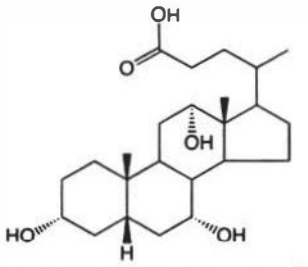
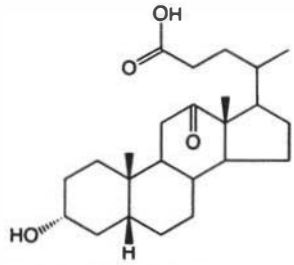
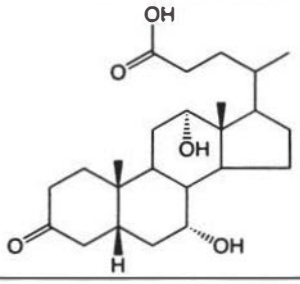
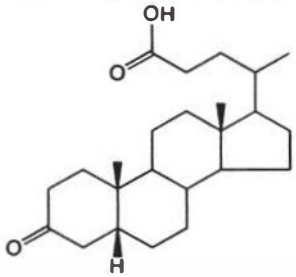
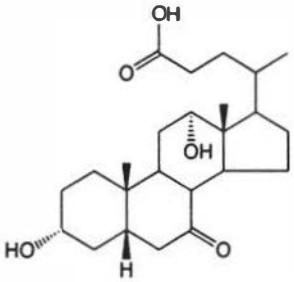
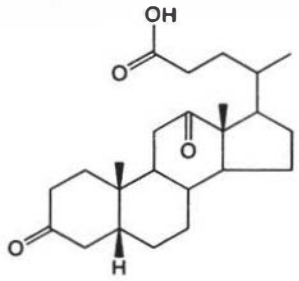
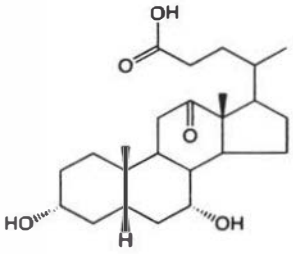
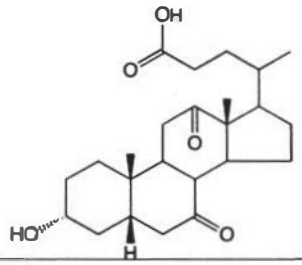
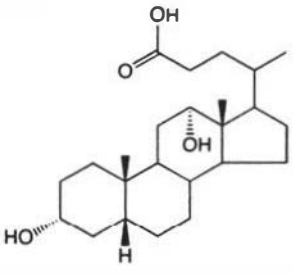
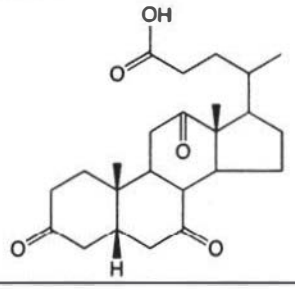


Fig 14. Metabolism of deoxycholic acid (DCA) and 12-oxolithocholic acid (12-oxoLCA) by *Clostridium scindens* ATCC 35704, *Clostridium hiranonis* DSM 13275, and *Clostridium scindens* VPI 12708. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lane: [1], *C. scindens* VPI with DCA; [2], *C. scindens* VPI with 12-oxoLCA; [3], *C. hiranonis* with DCA; [4], *C. hiranonis* with 12-oxoLCA; [5], 1 mM standard DCA; [6], 100 μM standard 12-oxoLCA.

Table 5. Oxo bile acids structures.

CA		12-oxoLCA	
3-oxoCA		3-oxoDCA	
7-oxoDCA		3,12-oxoDCA	
12-oxoCDCA		7, 12-oxoCA	
DCA		3, 7, 12-oxoCA	

Structure of the bile acids in this study. (CA), cholic acid; (3-oxoCA), 3-oxocholic acid; (7-oxoDCA), 7-oxodeoxycholic acid; (12-oxoCDCA), 12-oxochenodeoxycholic acid; (DCA), deoxycholic acid; (3-oxoDCA), 3-oxodeoxycholic acid; (12-oxoLCA), 12-oxolithocholic acid; (DCA), deoxycholic acid; (12-oxoCDCA), 12-oxochenodeoxycholic acid; (3, 12-dioxoLCA), 3, 12-dioxolithocholic acid; (7, 12-dioxoLCA), 7, 12-dioxolitocholic acid; (3, 7, 12-trioxoLCA), 3, 7, 12-trioxolithocholic acid.

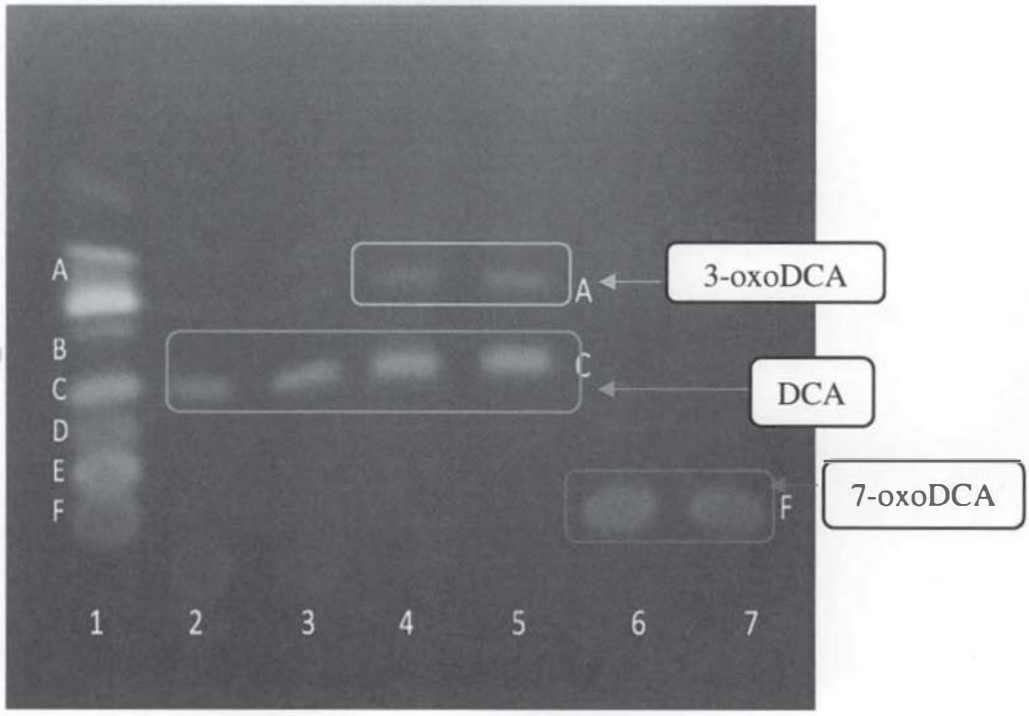


Fig 15. Metabolism of 7-oxodeoxycholic acid (7-oxoDCA) by *Clostridium scindens* ATCC 35704, *Clostridium hiranonis* DSM 13275, and *Clostridium hylemonae* DSM 15053. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM 7-oxoDCA, bile acids were extracted from cultures sterile brain heart infusion broth (BHI), dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12: 12: 1. Lanes: [1], standard bile acid mix containing: [A], (3-oxoDCA); [B], 12-oxolithocholic acid(12-oxoLCA); [C], deoxycholic acid(DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); and [F], 7-oxodeoxycholic acid (7-oxoDCA); [2, 3], *C. scindens*; [4, 5], *C. hiranonis*; and [6, 7], *C. hylemonae*.

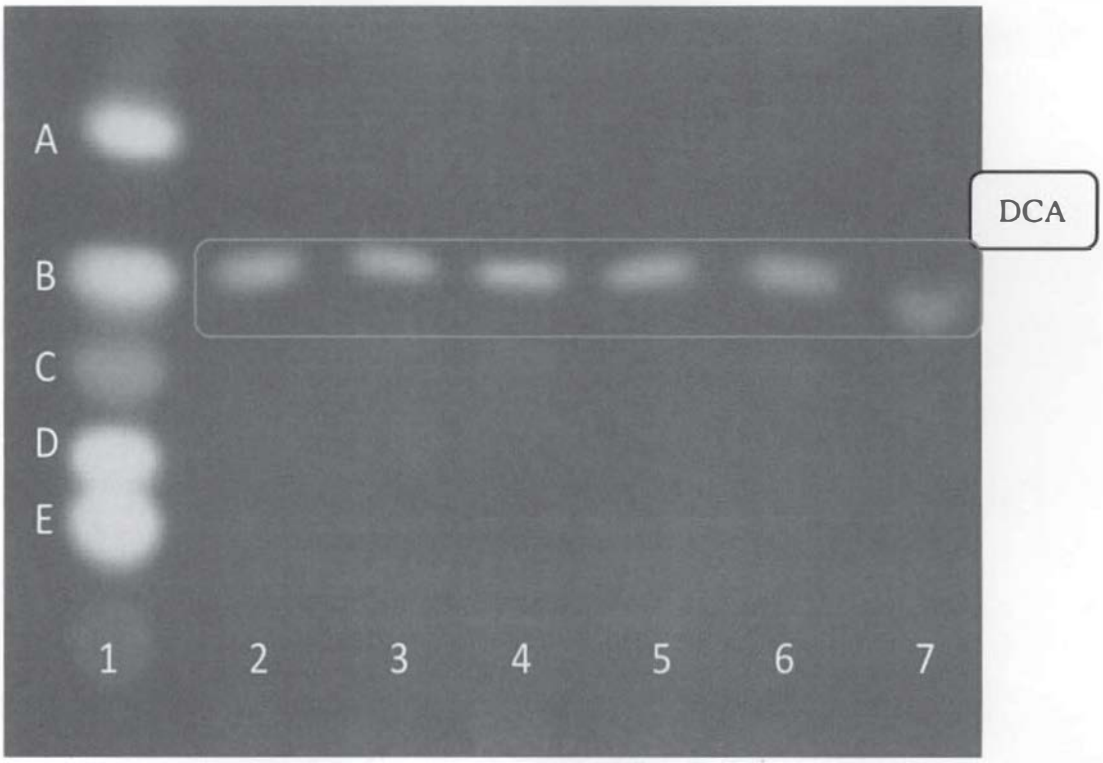


Fig 16. Metabolism of deoxycholic acid (DCA) by *Clostridium scindens* ATCC 35704, *Clostridium hiranonis* DSM 13275, and *Clostridium hylemonae* DSM 15053. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM DCA, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], standard bile acid mix containing : [A], 3-oxodeoxycholic acid (3-oxoDCA); [B], DCA; [C], 12-oxochenodeoxycholic acid (12-oxoCDCA); [D], 3-oxocholic acid (3-oxoCA); and [E], 7-oxodeoxycholic acid (7-oxoDCA); [2, 3], *C. scindens*; [4, 5], *C. hiranonis*; and , [6, 7], *C. hylemonae*.

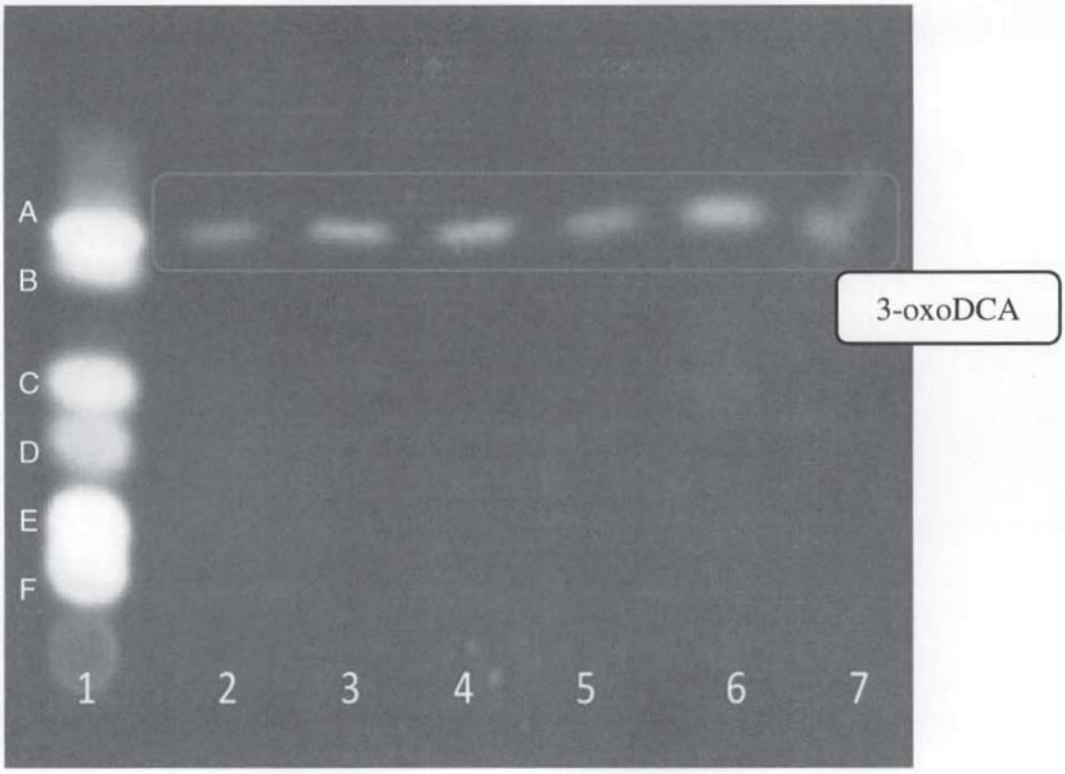


Fig 17. Metabolism of 3-oxodeoxycholic acid (3-oxoDCA) by *Clostridium scindens* ATCC 35704, *Clostridium hiranonis* DSM 13275, and *Clostridium hylemonae* DSM 15053. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM 3-oxoDCA, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], standard bile acid mix containing [A], 3-oxoDCA; [B], 12-oxolithocholic acid (12-oxo-LCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [2, 3], *C. scindens*; [4, 5], *C. hiranonis*; and [6, 7], *C. hylemonae*.

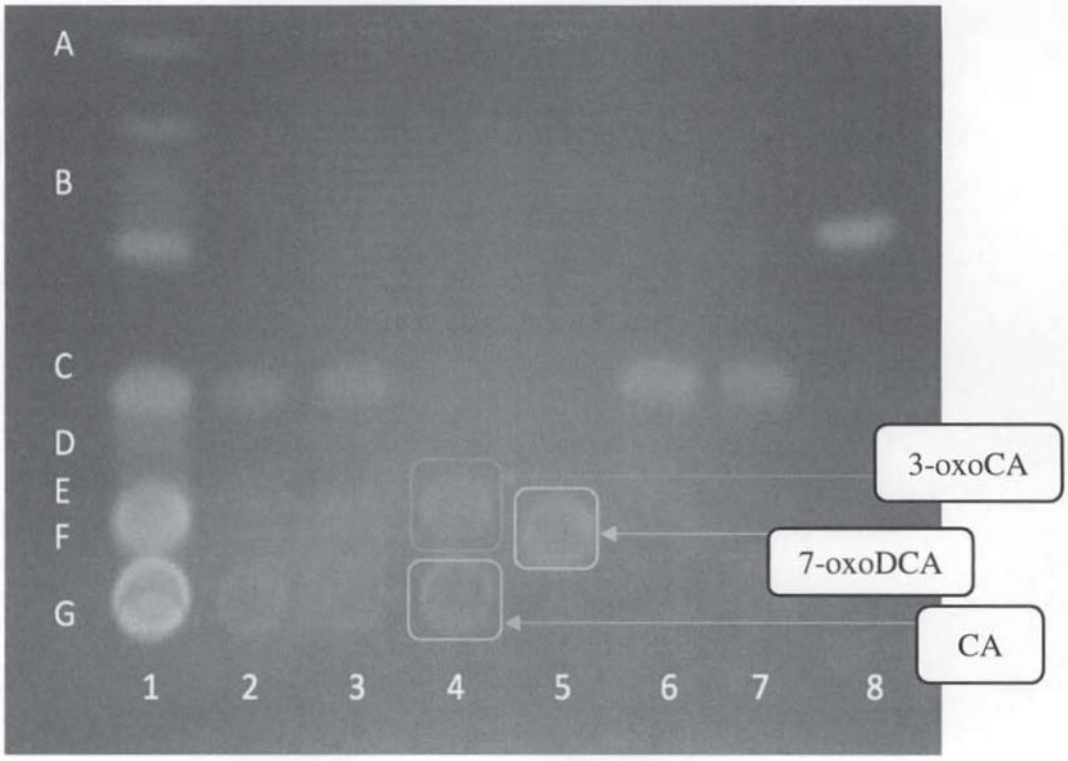


Fig 18. Metabolism of bile acids derivatives by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], standard bile acid mix containing: [A], 3-oxoDCA; [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], and 7-oxodeoxycholic acid (7-oxoDCA); [G]; cholic acid; [2], *C. scindens* with CA; [3], *C. scindens* with 7-oxo-DCA; [4], *C. scindens* with 3-ox-CA; [5], *C. scindens* with 12-oxoCDCA; [6], *C. scindens* with DCA; [7], *C. scindens* with 12-oxo-LCA; [8], *C. scindens* with 3-oxoDCA.

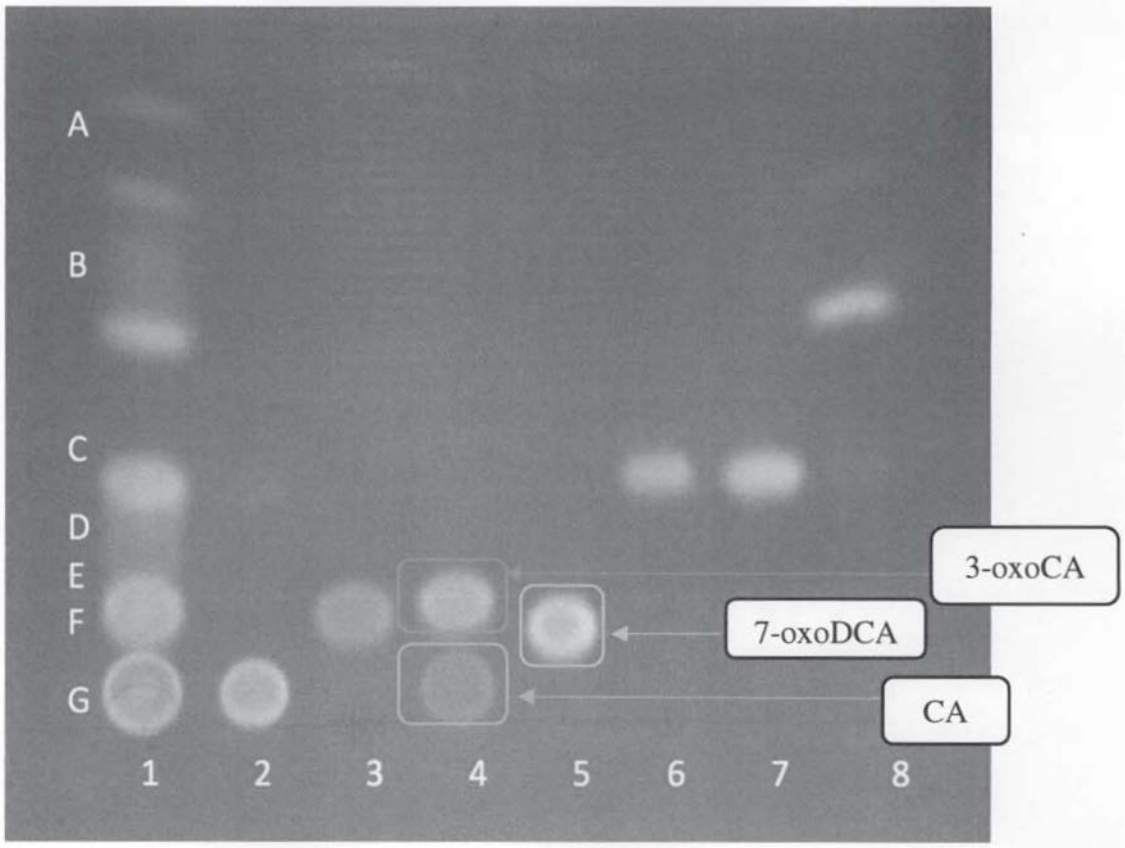


Fig 19. Metabolism of bile acids derivatives by *Clostridium hylemonae* DSM 15053. Organisms were grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], standard bile acid mix containing [A], 3-oxoDCA; [B], 12-oxolithocholic acid(12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxo-CDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [2], *C. hylemonae* with CA; [3], *C. hylemonae* with 7-oxoDCA; [4], *C. hylemonae* with 3-oxoCA; [5], *C. hylemonae* with 12-oxoCDCA; [6], *C. hylemonae* with DCA; [7], *C. hylemonae* with 12-oxoLCA; [8], *C. hylemonae* with 3-oxoDCA.

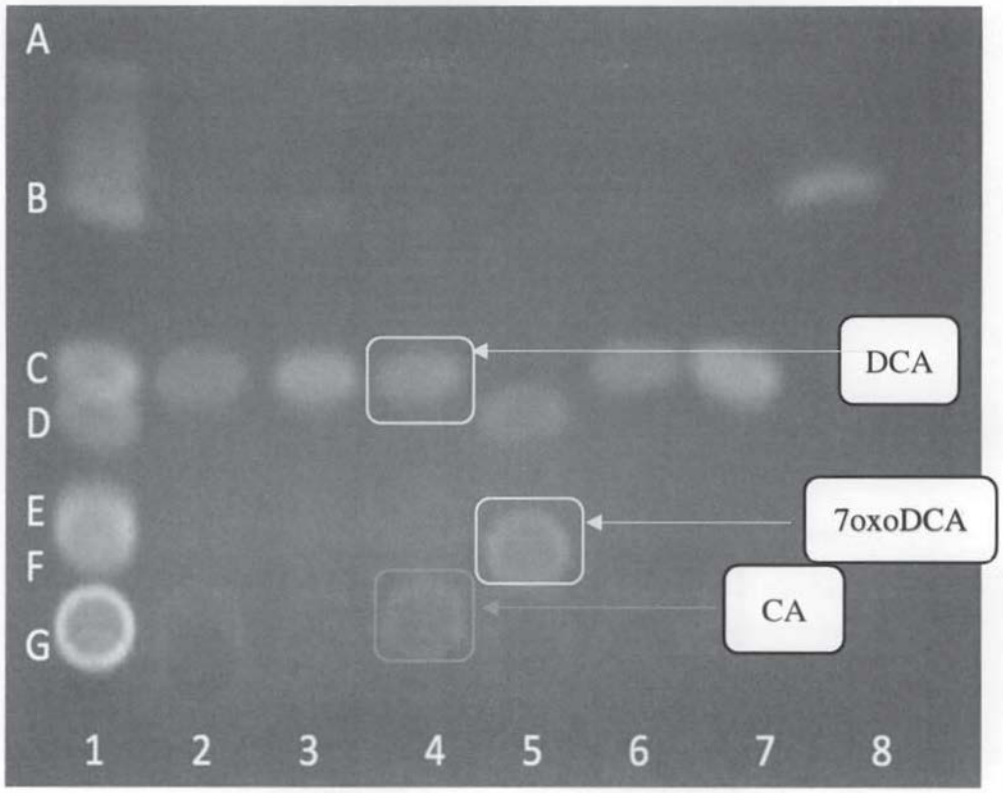


Fig 20. Metabolism of bile acids derivatives by *Clostridium hiranonis* DSM 13275. Organism was grown in brain heart infusion broth (BHI), bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: 1, 100 µM standard bile acid mix containing: [A], 3-oxodeoxycholic acid (3-oxo-DCA); [B], 12-oxolithocholic acid (12-oxoLCA) [C]; deoxycholic acid (DCA) [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); and [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); 2, *C. hiranonis* with CA; 3, *C. hiranonis* with 7-oxoDCA; 4, *C. hiranonis* with 3-oxoCA; 5, *C. hiranonis* with 12oxoCDCA; 6, *C. hiranonis* with DCA; 7, *C. hiranonis* with 12-oxoLCA; 8, *C. hiranonis* with 3-oxoDCA.

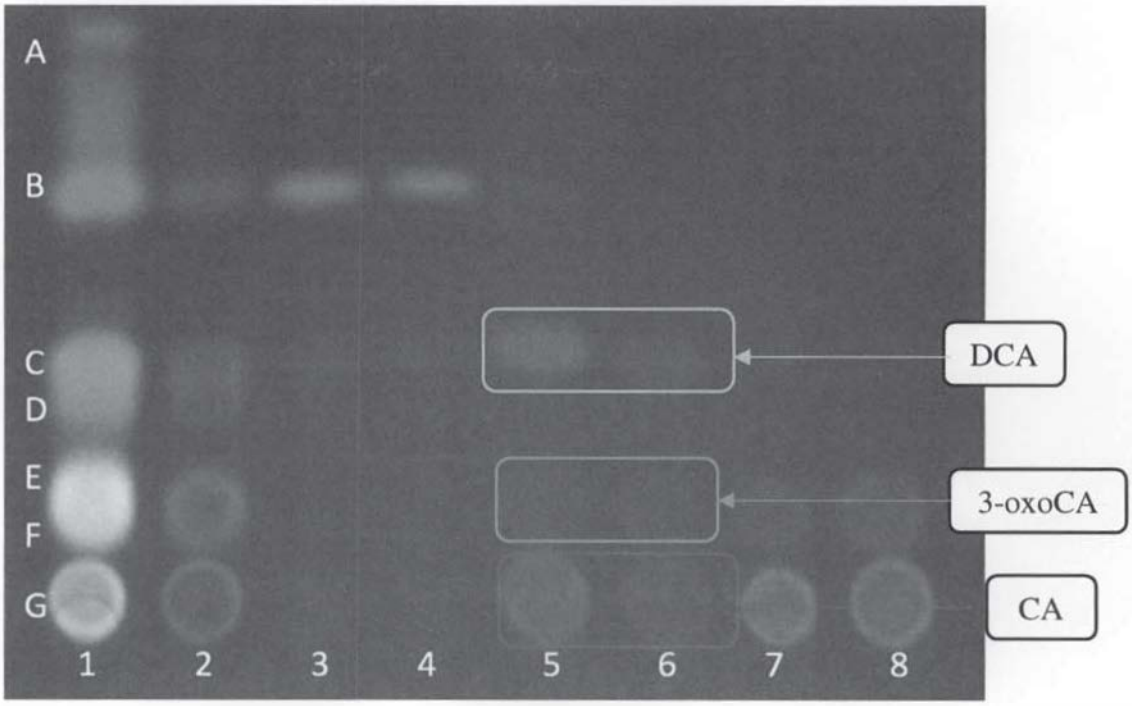


Fig 21. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolitocholic acid (7, 12-dioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion broth (BHI), supplemented with 100 μM bile acids, and bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix; [2], 100 μM standard bile acid mix containing : [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], *C. scindens* with 3, 12-dioxoLCA); [5, 6], *C. scindens* with 7, 12-dioxoLCA; [7, 8], *C. scindens* with 3, 7, 12-trioxoLCA).

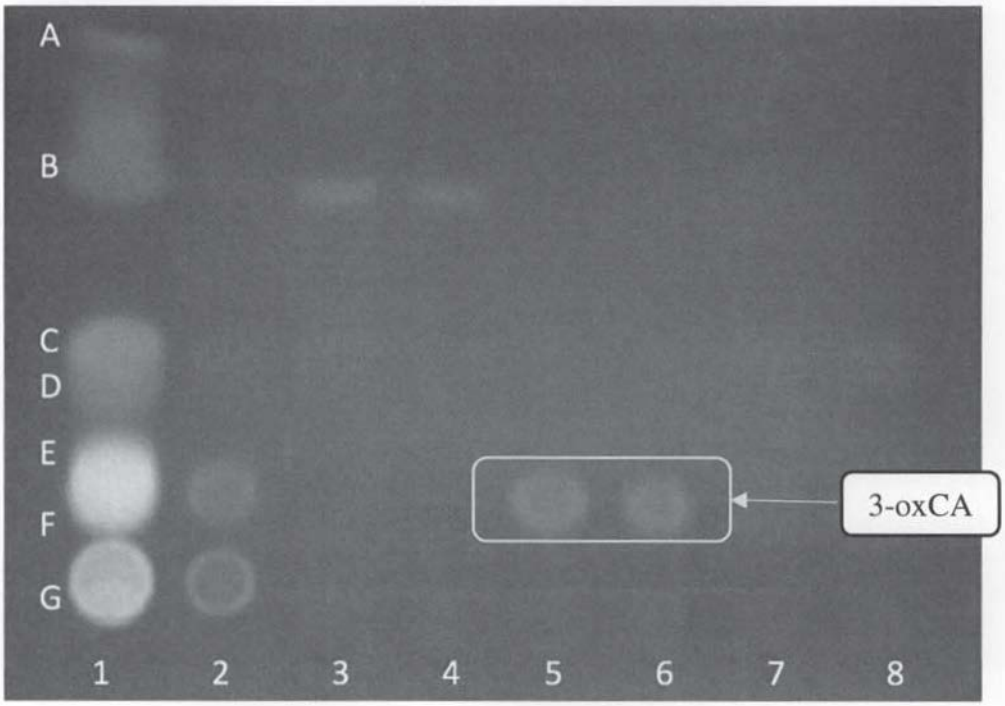


Fig 22. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolitocholic acid (7, 12-dioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium hylemonae* DSM 15053. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix; [2], 100 μM standard bile acid mix containing: [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxyholic acid (12-oxoCDCA); [E], 3-oxcholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], C. *hylemonae* with 3, 12-dioxoLCA; [5, 6], *C. hylemonae* with 7, 12-dioxoLCA; [7, 8], *C. hylemonae* with 3, 7, 12-trioxoLCA.

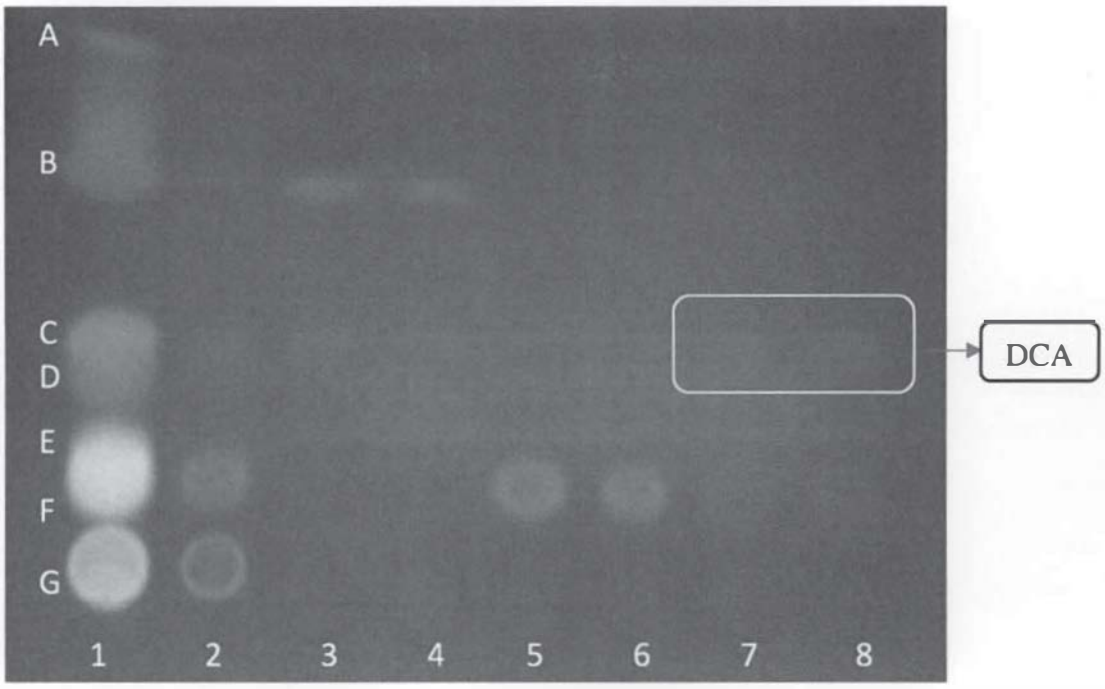


Fig 23. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolitocholic acid (7, 12-dioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium hylemonae* DSM 15053. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix; [2], 100 μM standard bile acid mix containing: [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], C. *hylemonae* with 3, 12-dioxoLCA); [5, 6], *C. hylemonae* with 7, 12-dioxoLCA); [7, 8], *C. hylemonae* with 3, 7, 12-trioxoLCA).

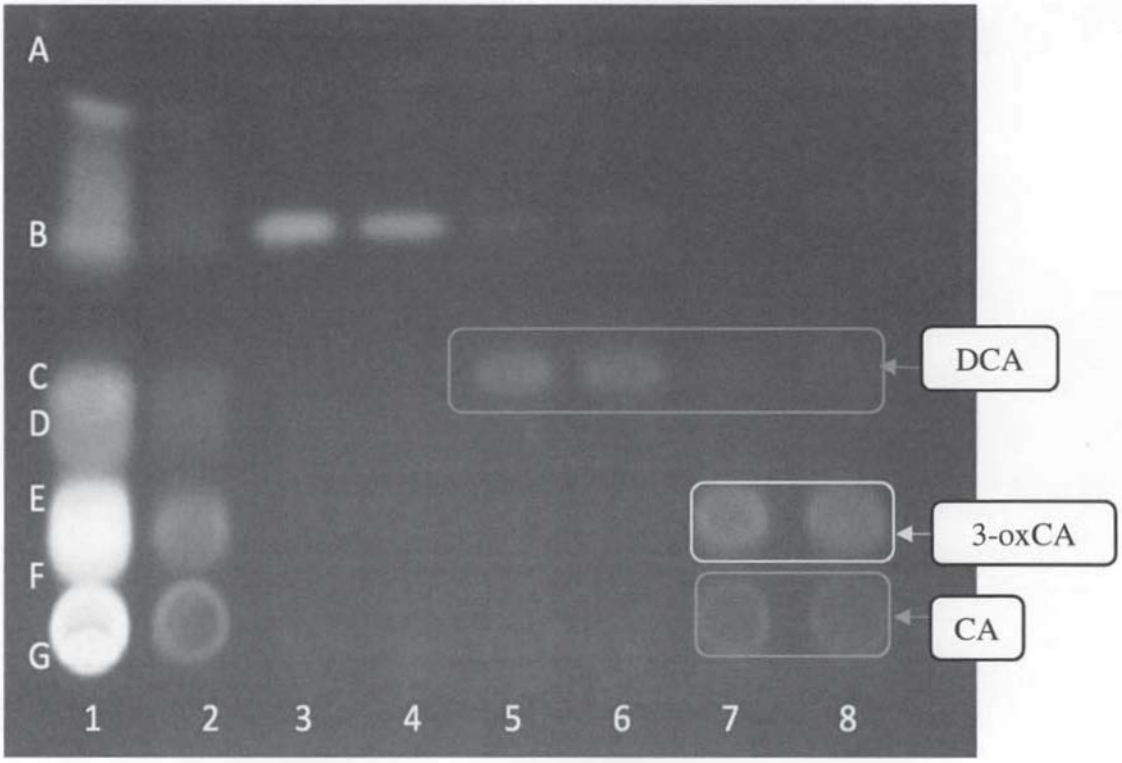


Fig 24. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolithocholic acid (7, 12-trioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium hiranonis* DSM 13275. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM of 3, 12-dioxoLCA; 7, 12-dioxoLCA; or 3, 7, 12-trioxoLCA, bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix , [2], 100 μM standard bile acid mix containing : [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], *C. hiranonis* with 3, 12-dioxoLCA; [5, 6], *C. hiranonis* with 7, 12-dioxoLCA; [7, 8], *C. hiranonis* with 3, 7, 12-trioxoLCA.

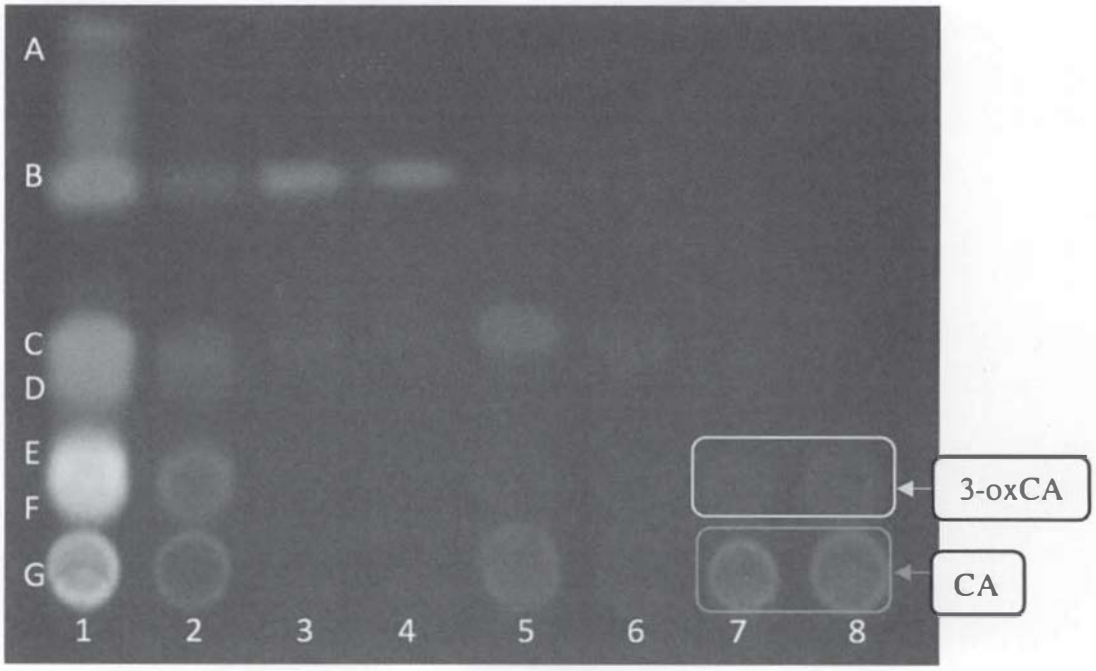


Fig 25. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolithocholic acid (7, 12-trioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix , [2], 100 μM standard bile acid mix containing : [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], *C. scindens* with 3, 12-dioxo-LCA; [5, 6], *C. scindens* with 7, 12-dioxoLCA; [7, 8], *C. scindens* with 3, 7, 12-trioxoLCA.

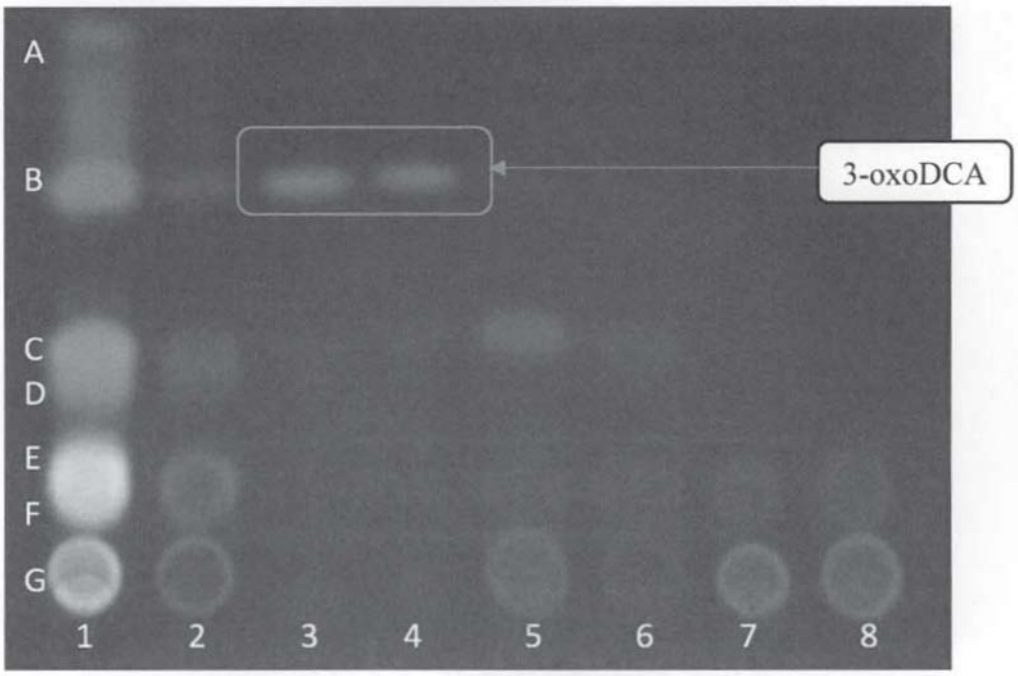


Fig 26. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolithocholic acid (7, 12-trioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 µM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix , [2], 100 µM standard bile acid mix containing : [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], *C. scindens* with 3, 12-dioxoLCA; [5, 6], *C. scindens* with 7, 12-dioxoLCA; [7, 8], *C. scindens* with 3, 7, 12-trioxoLCA.

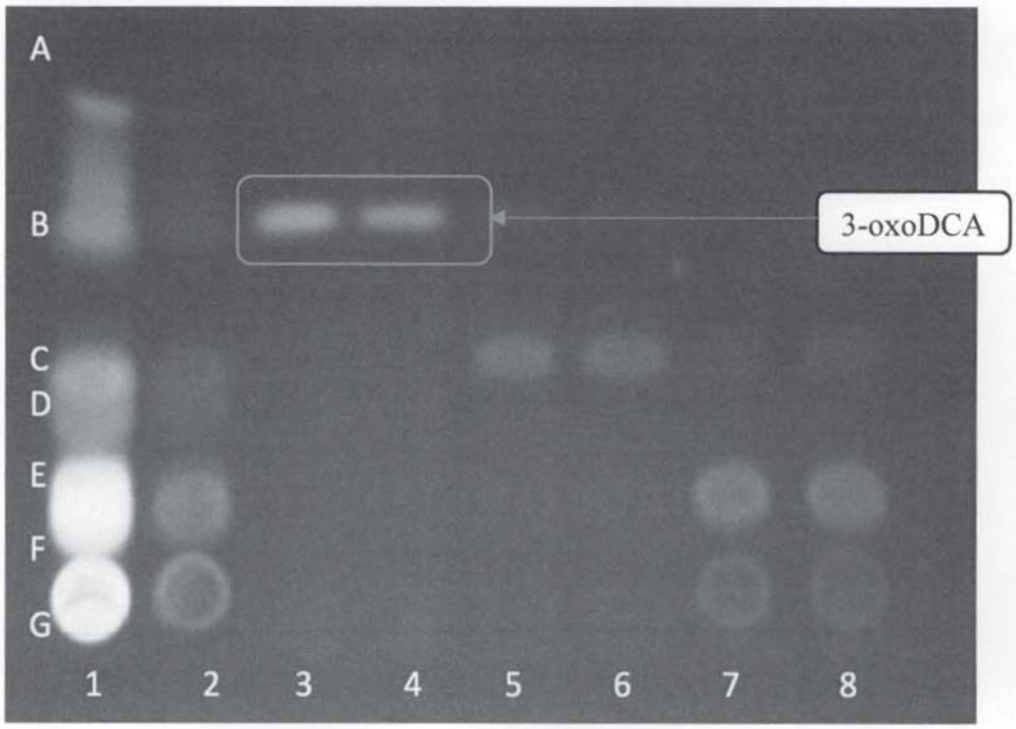


Fig 27. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolitocholic acid (7, 12-dioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium hiranonis* DSM 13275. Organism was grown in brain heart infusion broth (BHI), supplemented with 100 µM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix; [2], 100 µM standard bile acid mix containing: [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], C *hiranonis* with 3, 12-dioxoLCA; [5, 6], *C. hiranonis* with 7, 12-dioxoLCA; [7, 8], *C. hiranonis* with 3, 7, 12-trioxoLCA.

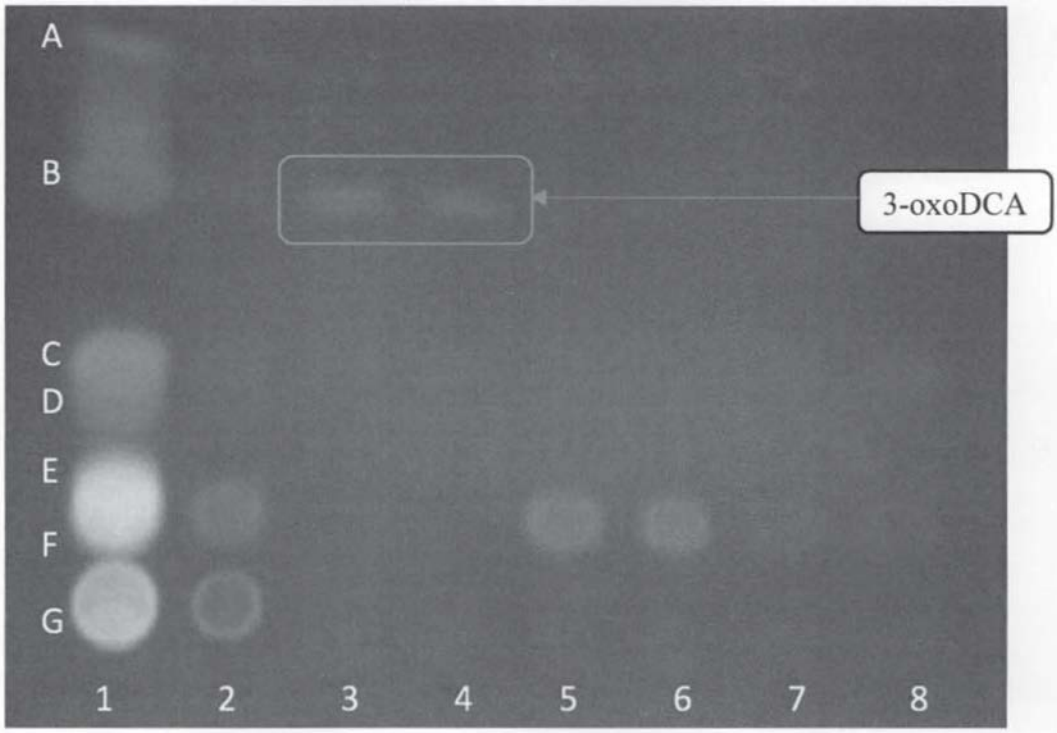


Fig 28. Metabolism of [3, 12-dioxolithocholic acid (3, 12-dioxoLCA); 7, 12-dioxolitocholic acid (7, 12-dioxoLCA); 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA)] by *Clostridium hylemonae* DSM 15053. Organism was grown in brain heart infusion broth (BHI) supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 1 mM standard bile acid mix; [2], 100 μM standard bile acid mix containing: [A], 3-oxodeoxycholic acid(3-oxoDCA); [B], 12-oxolithocholic acid (12-oxoLCA); [C], deoxycholic acid (DCA); [D], 12-oxochenodeoxycholic acid (12-oxoCDCA); [E], 3-oxocholic acid (3-oxoCA); [F], 7-oxodeoxycholic acid (7-oxoDCA); [G], cholic acid(CA); [3, 4], C. *hylemonae* with 3, 12-dioxoLCA); [5, 6], *C. hylemonae* with 7, 12-dioxoLCA); [7, 8], *C. hylemonae* with 3, 7, 12-trioxoLCA).

Metabolism of bile acid combinations. (i) CA and DCA. A combination of CA and DCA, each at a final concentration of 0.1 mM, were added to BHI broth to evaluate the ability of these organisms (i) to dehydroxylate CA in the presence of DCA and (ii) to produce any possible novel products from CA or DCA metabolism. *C. scindens* ATCC 35704, *C. scindens* VPI 12708, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 were all incapable of metabolizing DCA (Figure 16). Thus, it was difficult to determine whether the combination of CA and DCA suppressed the conversion of CA to DCA to any notable degree given the qualitative nature of TLC analysis. However, *C. scindens* ATCC 35704 and *C. hiranonis* DSM 13275 cultures did reveal a faint spot of CA upon TLC analysis which indicated that both organisms transformed CA partially to DCA (Figure 29). *C. hiranonis* DSM 13275 formed other unknown products beside CA and DCA, and it was difficult to identify the unknown products via TLC only (Figure 29). *C. hylemonae* DSM 15053 cultures yielded a darker, more intense CA spot on the TLC plate while the spot intensity for DCA was similar to the DCA standard, which also indicated the weak activity of *C. hylemonae* DSM 15053 to generate DCA from CA (Figure 29). Considerably, *C. scindens* VPI 12708 appeared to convert most, if not all, of the CA to make DCA without the generation of any unknown product(s) (Figure 29). However, *C. scindens* VPI 12708 was eliminated from further experiments in the present study due to its inability to transform 12-oxoLCA to DCA (Figure 9).

(ii) CA and 12-oxoLCA. Another combination of bile acid consisting of CA and 12-oxoLCA was applied in this study to examine another aspect of dehydroxylation/reduction by determining if a mixture of bile acids was metabolized differently than when a single bile acid was added to BHI broth. To examine this possibility, two culture conditions were

tested. First, to evaluate if CA induction impacted the bioconversion of CA/12-oxoLCA mixture, cells were initially cultured with CA (1st passage) and then transferred to BHI broth supplemented with both CA and 12-oxoLCA (2nd passage). The second culture condition examined was the bioconversion of CA/12-oxoLCA mixture directly without prior induction of cells with CA. Under both conditions, *C. scindens* ATCC 35704 revealed the same products, DCA, CA, 3-oxoDCA, and an unknown product (Figure 30). This unknown product appeared to be similar to 3-oxoCA, 7-oxoDCA, or 12-oxoLCA (Figure 30).

(iii) CA and 12-oxoCDCA. A combination of CA and 12-oxoCDCA was also examined using the same experimental approach as described above for the CA/12-oxoLCA mix. In these experiments, C. *scindens* produced DCA and 7-oxoDCA under both culture conditions (induced/non-induced cells) (Figure 31). An undetermined product similar to 3-oxoDCA was also formed under both culture conditions (Figure 31).

For *C. hiranonis*, cells actively formed DCA in BHI supplemented with the CA/12-oxoCDCA mix (Figure 32). The DCA was apparently produced from CA dehydroxylation since 12-oxoCDCA was metabolized very little, if at all, by either induced or non-induced cells. 3-oxoCA, and 3-oxoDCA were identified as additional products under both culture conditions (Figure 32).

Interestingly, C. *hylemonae* DSM 15053 completely consumed 12-oxoCDCA under both culture conditions (induced/non-induced cells) (Figure 33). Based on the results shown in Table 4 for individual bile acids, C. *hylemonae* DSM 15053 has the capacity to convert 12-oxoCDCA solely to 7-oxoDCA (Table 4). 7-oxoDCA did not seem to be metabolized further by *C. hylemonae* DSM 15053 (Table 4). With non-induced cells, CA was not appreciably dehydroxylated to DCA (Figure 33; Table 4). In sharp contrast,

induced cells of *C. hylemonae* DSM 15053 appeared to have more metabolic capabilities than noninduced cells. For example, induced cells were able to readily produce DCA, apparently from CA, although it is also possible that some of the DCA was derived from 7-oxoDCA (e.g., requiring oxo-group reduction and dehydroxylation at the C-7 position) or 12-oxoCDCA (e.g., requiring dehydroxylation at the C-7 position and oxo-group reduction at the C-12 position). In this regard, induced cells, unlike non-induced cells, were able to produce 12-oxoLCA from the CA/12-oxoCDCA mixture, with 12-oxoCDCA a likely candidate. However, the ultimate bile acid source(s) for 12-oxoLCA formation remain to be determined as well as the reason for 12-oxoLCA accumulation when 12-oxoLCA reduction to DCA is possible (Figure 33). Nonetheless, these findings seem to suggest that for some bile acid-metabolizing bacteria hydroxyl-group removal from oxo-bile acids, like non-oxo-bile acids, is an inducible metabolic feature. Such potential metabolic potentials will require further studies in *C. hylemonae* DSM 15053 and other bile acid-dehydroxylating bacteria.

Potential regulatory substrates for 12-oxoLCA reduction to DCA. Hypothetically, it was important to examine the impact of potential electron acceptors thiosulfate ($S_2O_3^{2-}$) and nitrate (NO_3^-) or potential electron donors such as hydrogen gas (H_2), pyruvic acid ($C_3H_4O_3$), and formate (HCO_2^-) on the conversion of 12-oxoLCA to DCA. It should be noted that (H_2), pyruvic acid ($C_3H_4O_3$), and formate (HCO_2^-) are also end products formed during glucose fermentation by *C. scindens* ATCC 35704 (40, 41) Overall, the presence of these potential electron acceptors or electron donors did not appear to impact the conversion of 12-oxoLCA to DCA by *C. scindens* ATCC 35704 during growth (Figure 34).

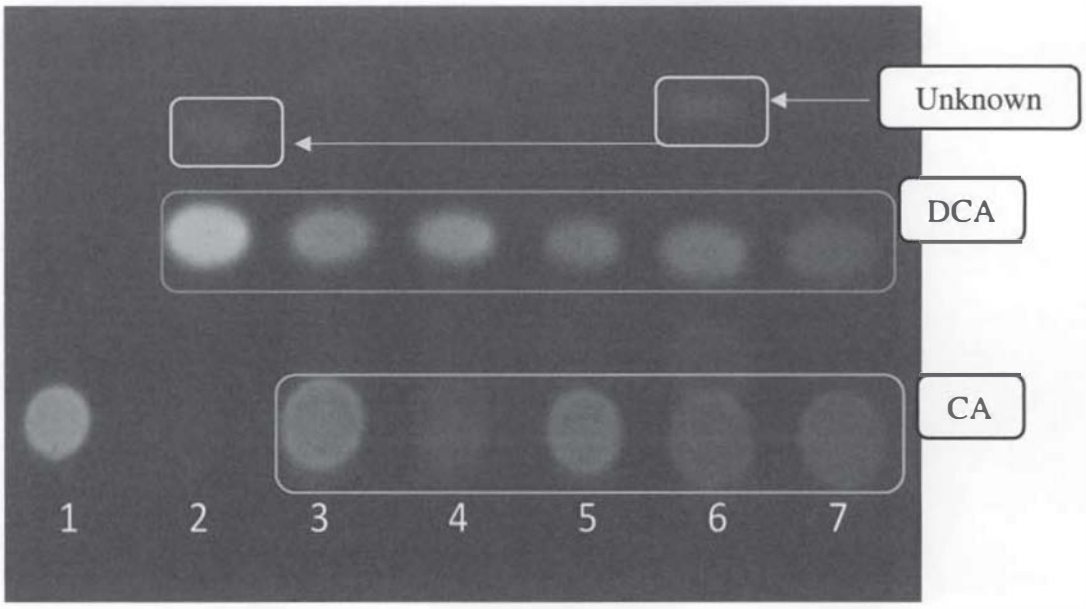


Fig 29. Metabolism of mix bile acid containing cholic acid (CA) and deoxycholic acid (DCA) by *Clostridium hylemonae* DSM 15053, *Clostridium scindens* VPI 12708, *Clostridium paraputrificum*, *Clostridium hiranonis* DSM 13275, and *Clostridium scindens* ATCC 35704. Organism were grown in brain heart infusion broth (BHI), supplemented with 100 µM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lane: [1], 100 µM of standard CA; 2, 100 µM of standard DCA; 3, *C. hylemonae* DSM 15053 with CA/DCA; 4, *C. scindens* VPI 12708 with CA/DCA; 5, *C. paraputrificum* with CA/DCA; 6, *C. hiranonis* DSM 13275 with CA/DCA; and 7, *C. scindens* ATCC 35704 with CA/DCA.

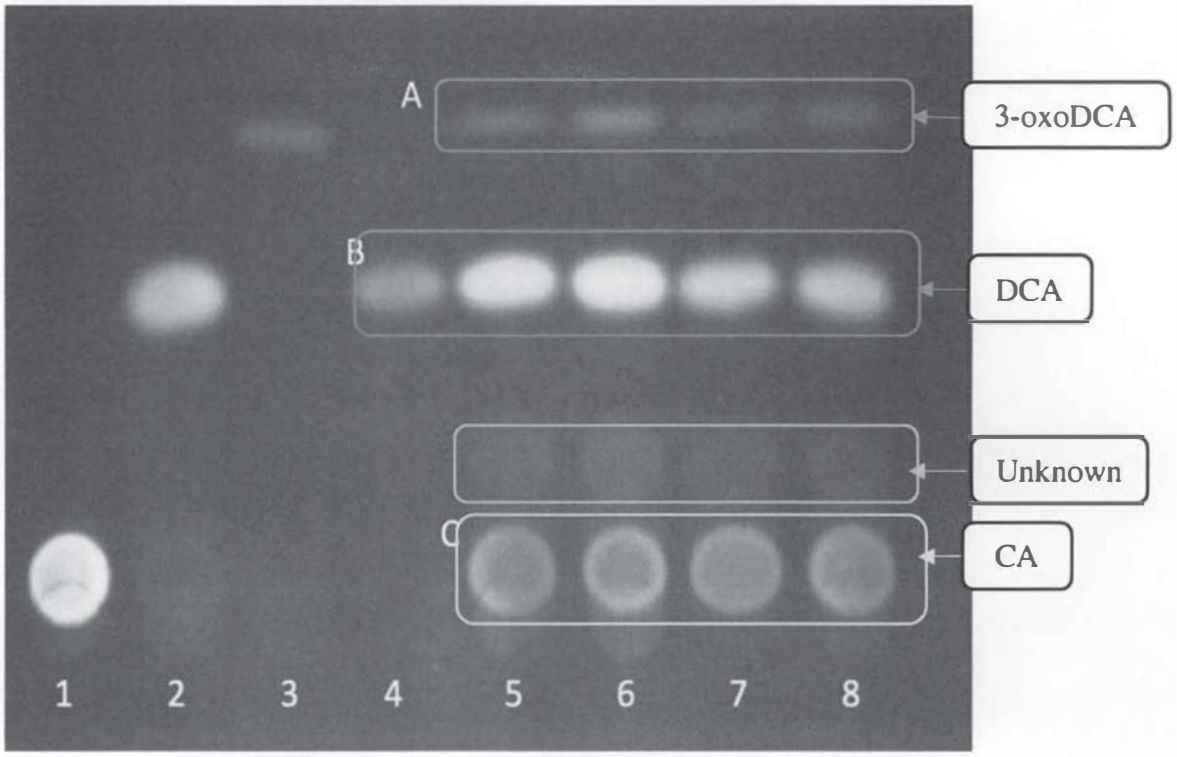


Fig 30. Metabolism of mix bile acids containing cholic acid (CA) and 12-oxolithocholic acid (12-oxoLCA) by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion (BHI) broth supplemented with 100 µM bile acids Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid; 12:12:1. Lanes: [1], standard CA; [2], standard deoxycholic acid (DCA); [3], standard 12-oxoLCA; [4], *C. scindens* with 12-oxoLCA; [5, 6], *C. scindens* induced with CA for overnight prior to inculcated with 12-oxoLCA; [7, 8], *C. scindens* with combination of (CA/12-oxoCDCA) at final concentration of 100 µM; [A], 3-oxolithocholic acid (3-oxoDCA); [B], deoxycholic acid (DCA) ; [C] cholic acid (CA).

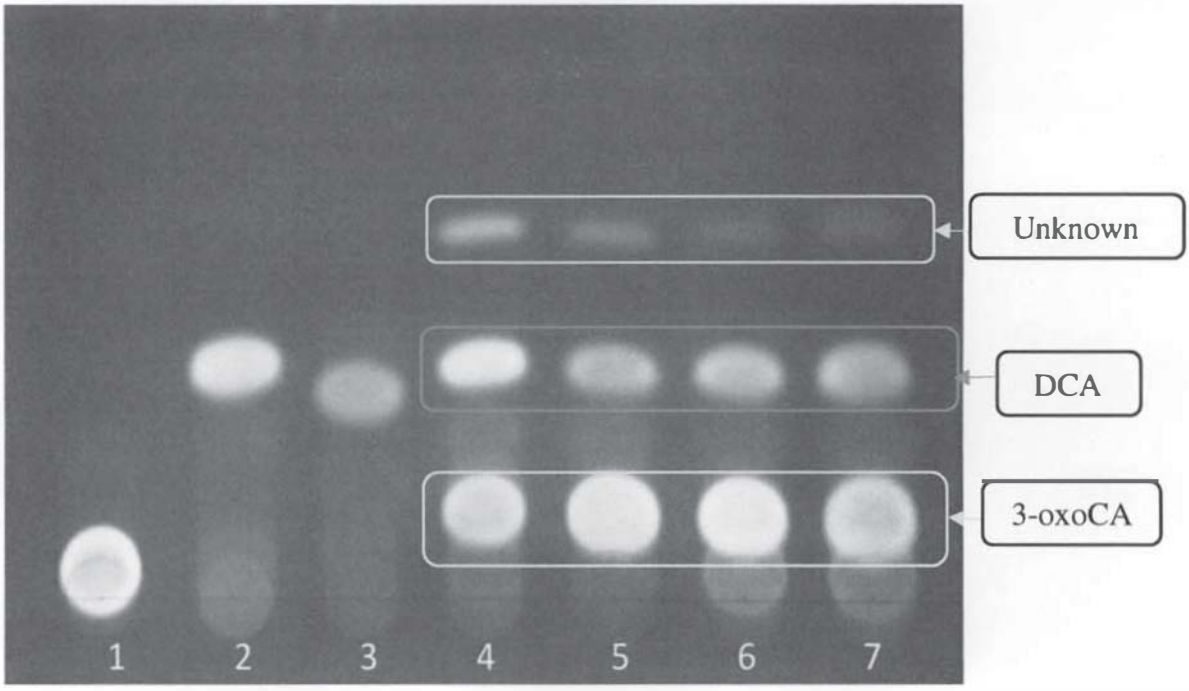


Fig 31. Metabolism of a combination of cholic acid (CA) and 12-oxochenodeoxycholic acid (12-oxoCDCA) by *Clostridium scindens* ATCC 35704. Organism was grown in brain heart infusion (BHI) broth supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 50 μM standard CA; [2], standard deoxycholic acid (DCA); [3], standard 12-oxoCDCA; [4, 5], 1st passage of *C. scindens* with CA/12-oxoCDCA; and [6, 7], 2nd passage of *C. scindens* with CA/12-oxoCDCA.

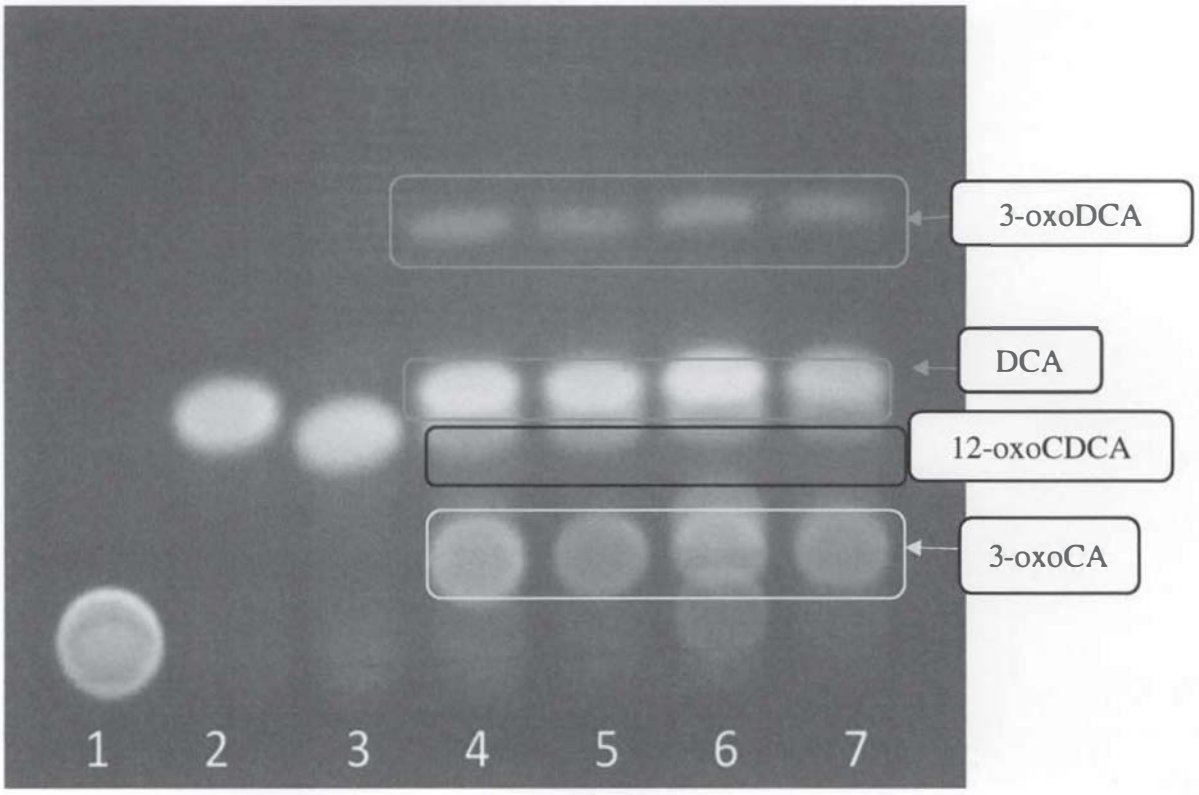


Fig 32. Metabolism of a combination of cholic acid (CA) and 12- oxochenodeoxycholic acid (12-oxoCDCA) by *Clostridium hiranonis* DSM 13275. Organism was grown in brain heart infusion broth (BHI), supplemented with 100 µM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12: 12: 1. Lanes: [1], 50 µM standard CA; [2], standard deoxycholic acid (DCA); [3], standard 12-oxoCDCA; [4, 5], 1stpassage of *C. hiranonis* with CA/12-oxoCDCA; [6, 7], 2nd passage of *hiranonis* with CA/12-oxoCDCA.

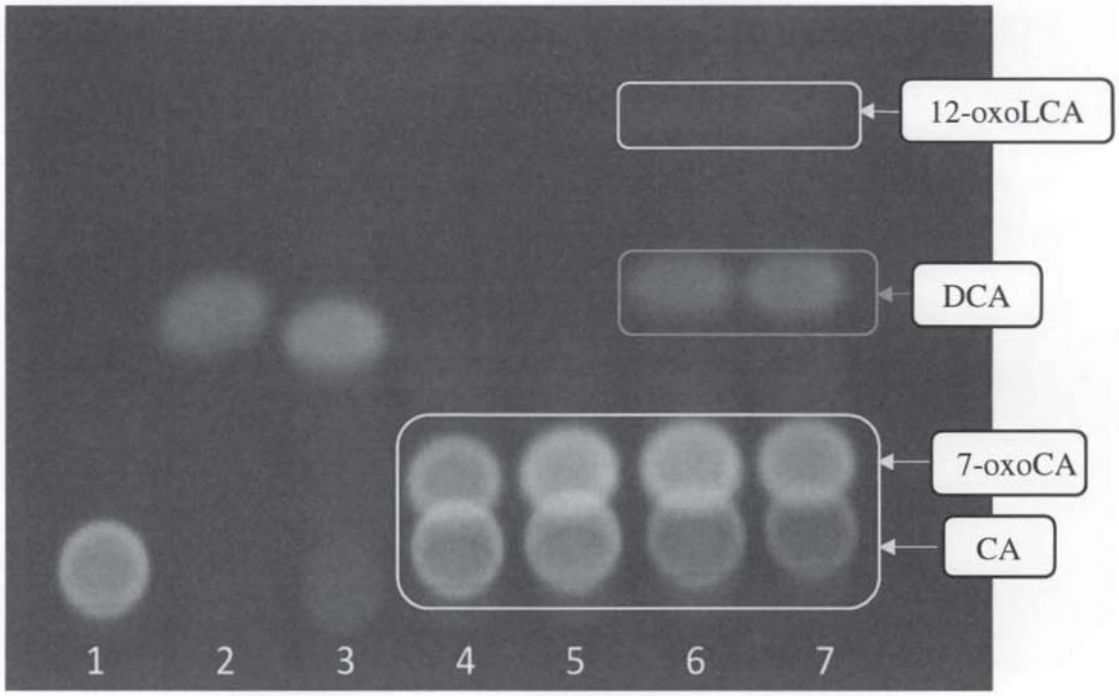


Fig 33. Metabolism of a combination of cholic acid (CA) and 12-oxochenodeoxycholic acid (12-oxoCDCA) by *Clostridium hylemonae* DSM 15053. Organism was grown in brain heart infusion broth (BHI), supplemented with 100 μM bile acids. Bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], 50μM standard CA; [2], standard deoxycholic acid (DCA); [3], standard 12-oxoCDCA; [4,5], 1st passage of *C. hylemonae* with CA/12-oxoCDCA; and [6,7], 2nd passage of *hylemonae* with CA/12-oxoCDCA.

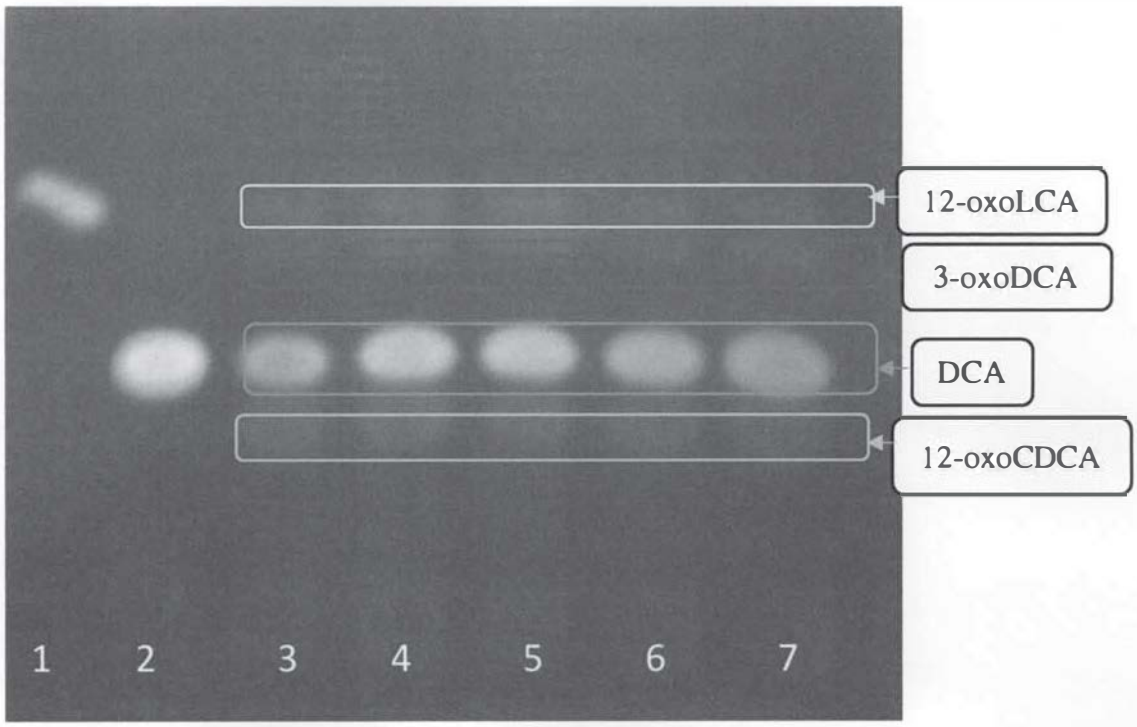


Fig 34. Metabolism of 12-oxolithocholic acid (12-oxoLCA) by *Clostridium scindens* ATCC 35704 in the presence of potential regulatory substrates. Organism was grown in brain heart infusion broth (BHI), each substrate (10 mM final concentration) was added separately to each culture supplemented with 100 μM 12-oxoLCA, and bile acids were extracted from cultures, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid; 12:12:1. Lanes: [1] standard 12-oxolithocholic acid (12-oxoLCA); [2], standard deoxycholic acid (DCA); [3], *C. scindens* with thiosulfate (S_2O_3); [4], *C. scindens* with nitrate (NO_3^-); [5], *C. scindens* with pyruvic acid ($C_3H_4O_3$); [6], *C. scindens* with hydrogen gas (H_2); and [7], *C. scindens* with formate (HCO_2^-).

Detection limit of bile acids. It was extremely important to determine the lowest detectable concentration of each bile acid used in this study. Knowing the minimal detectable amount allows better identification of products (fewer false negatives) and unspecified products (fewer false positives). Different concentrations of bile acids were added to BHI broth, extracted, and then analyzed by TLC using different solvent systems depending on the type of bile acid (e.g., mono; di; and tri) added to the medium. Consequently, the lowest amount of each bile acid was determined to be ≤ 10 μM in BHI broth (Figure 35-36). These results indicated that most, if not all, possible products from each bile acid transformation would be identifiable using the TLC method employed in this study. Despite the fact that some products were hard to identify without examining the corresponding standard on the same TLC plate, the low detection limit provided a high degree of confidence that the bile acid substrates and transformation products from bile acids would be measurable in BHI cultures used in this study (Figure 35-36).

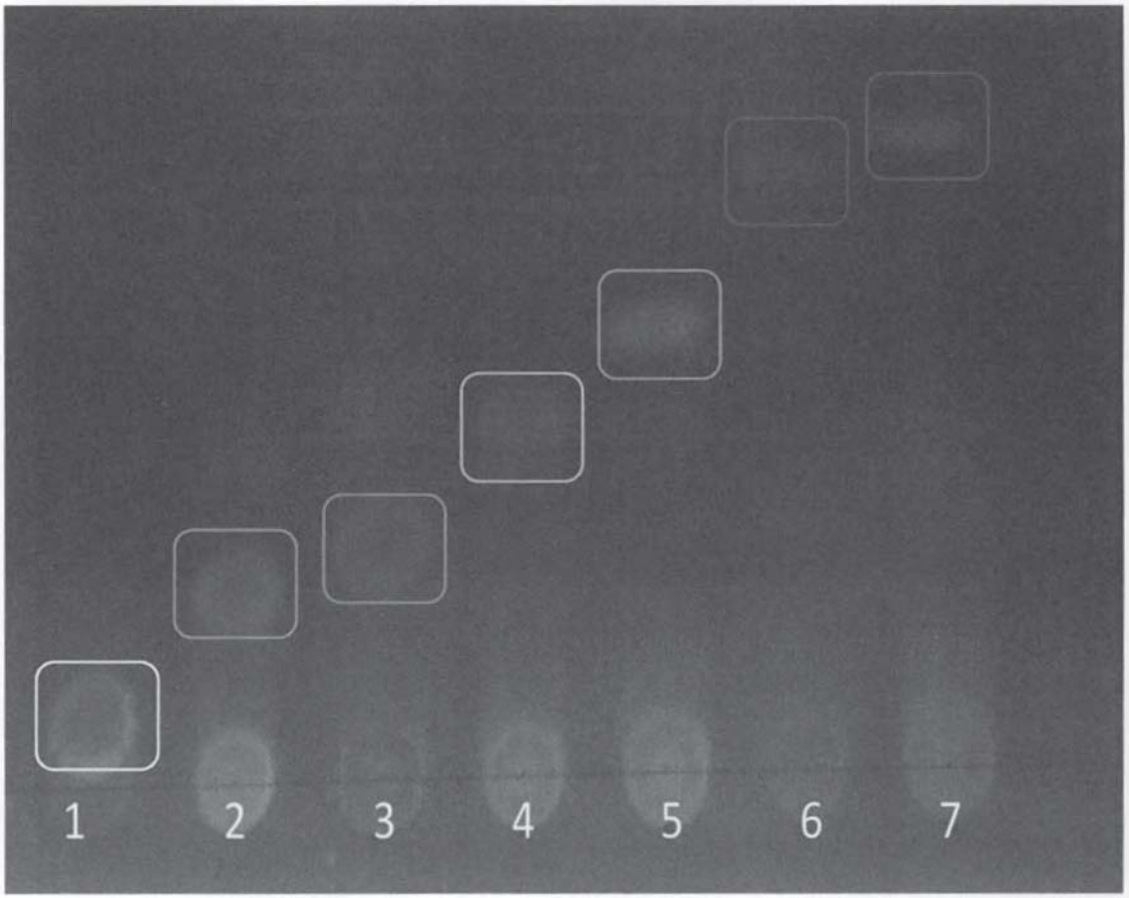


Fig 35. TLC analysis of (10 µM) mono-bile acid standards in methanol. Bile acids were extracted from sterile brain heart infusion (BHI) broth supplemented with 10 µM bile acids, bile acids were extracted from BHI medium, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 µm thick, non-UV, TLC plate. Solvent system used to separate bile acids was ethyl acetate, cyclohexane, and glacial acetic acid in a ratio of 12:12:1. Lanes: [1], cholic acid (CA); [2], 7-oxodeoxycholic acid (7-oxoDCA); [3], 3-oxocholic acid (3-oxoCA); [4], 12-oxochenodeoxycholic acid (12-oxo-CDCA); [5], deoxycholic acid (DCA); [6], 12-oxolithocholic acid (12-oxoLCA); [7], 3-oxodeoxycholic acid (3-oxoDCA).

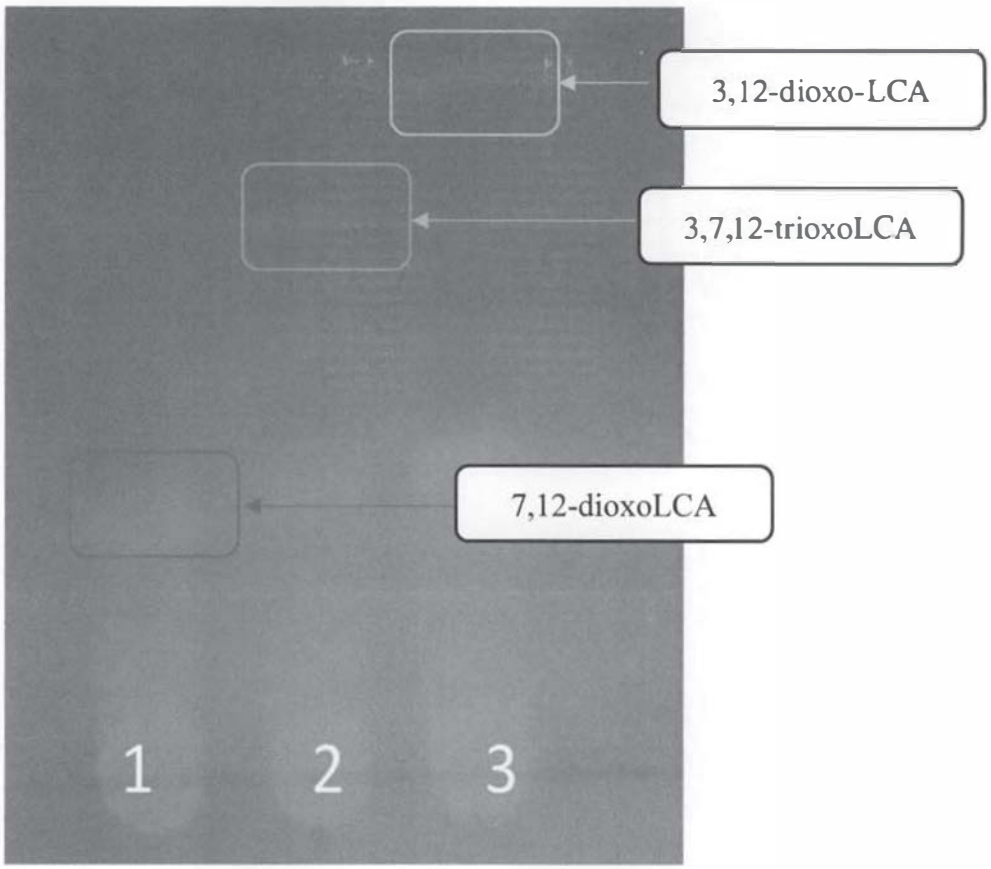


Fig 36. TLC analysis of (10 μM) di and tri bile acids standards in methanol. Bile acids were extracted from sterile brain heart infusion (BHI) broth supplemented with 10 μM of bile acids, bile acids were extracted from BHI medium, dried, re-suspended in methanol, and spotted on a 20-cm x 20-cm, AL SIL G, 250 μm thick, non-UV, TLC plate. Solvent system used to separate bile acids was toluene, 1-4 dioxane, and glacial acetic acid in a ratio of 70:20:2. Lane [1], 7,12-dioxolitocholic acid (7,12-dioxoLCA); [2], 3, 7, 12-trioxolithocholic acid (3, 7, 12-trioxoLCA); [3], 3, 12-dioxolithocholic acid (3, 12-dioxoLCA).

Gene cloning and expression. (i) DNA amplification. Genomic DNA from *C. scindens* ATCC 35704 was provided by University of Illinois at Urbana-Champaign UIUC to amplify the putative 12α-HSDH gene from *C. scindens* ATCC 35704. Cloning primers were used during the polymerase chain reaction (PCR) process in order to provide amplified 12α-HSDH copies for insertion into the cloning vector. It was important to assure the presence of the correct gene among the DNA amplification processes, and, to ensure that, gel electrophoresis was applied to detect DNA size and to confirm the correctness of the amplification, gene was successfully cloned and amplified for gene expression, recombinant 12α-HSDH gene was visualized in a pure band and correct size compared to the digested vector (pET51), with the proper restriction enzyme, and compared also with the undigested vector (Figure 37).

(ii) Expression of recombinant protein. 12α-HSDH gene was successfully inserted into the vector *E. coli* cells BL21 CodonPlus, transformed with pET51b plasmid containing cloned gene of interest, 12α-HSDH, into *C. scindens*. Inserted plasmid was followed by gel electrophoresis to identify the correct insertion of the plasmid (Figure 38). The selected plasmid from *C. scindens* was effectively sequenced at Carl R. Woese Institute for Genomic Biology at UIUC. Putative 12α-HSDH gene sequence from *C. scindens* was aligned with the amino acid sequence of the 12α-HSDH gene from *Clostridium* spp. ATCC 29733 (ERJ00208.1). The nucleotide sequence of 12α-HSDH from *C. scindens* confirmed that there were no point mutations before protein expression was done. (Table 6).

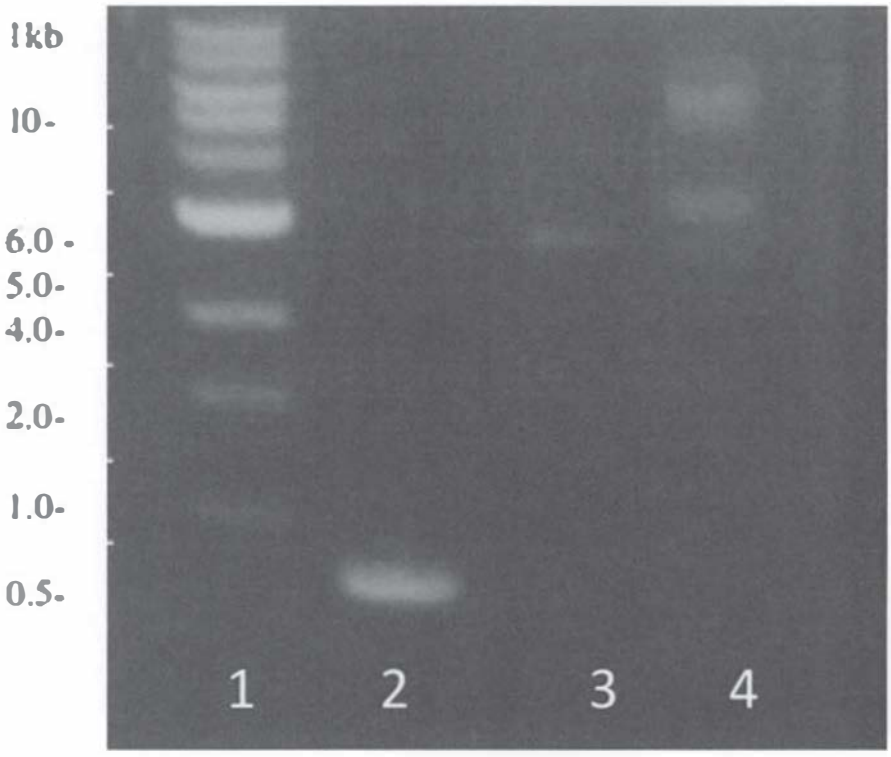


Fig 37. Gel electrophoresis image of the polymer chain reaction (PCR) with primers containing overhang for 12α-HSDH inserted into *Clostridium scindens* ATCC 35704. Lane: [1], 1-kb DNA ladder; [2], 12α-HSDH from *C. scindens*; [3], digested vector (pET51) with the restriction enzymes; and [4], undigested vector (pET51).

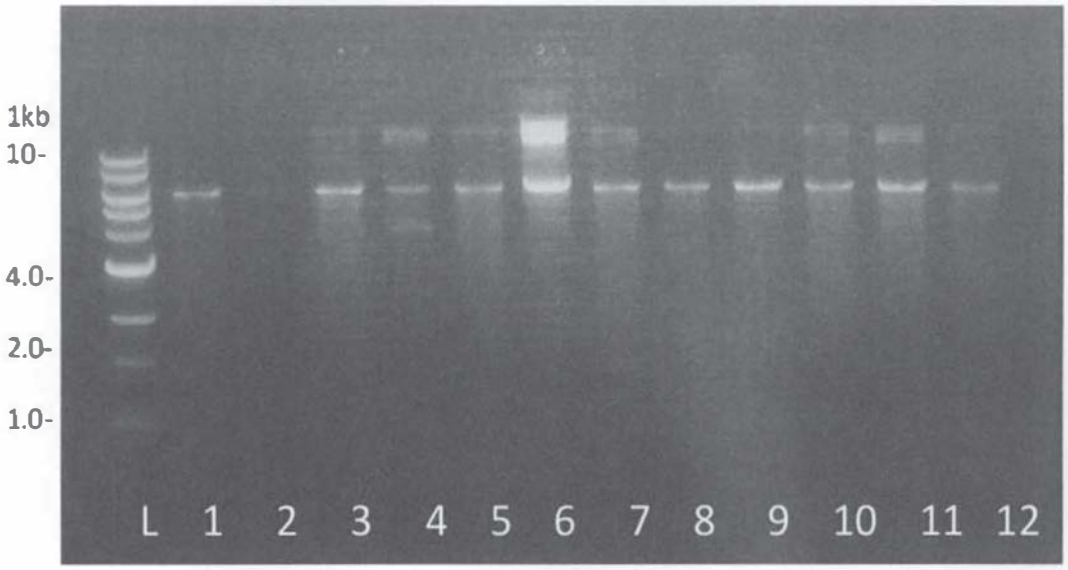


Fig 38. Gel electrophoresis image of the transformants plasmid *E. coli* DH5α containing 12α-HSDH from *Clostridium scindens* ATCC 35704. Lane [1]; 1-Kb DNA ladder; [1, 3, 5, 8, and 9]; contain the correct size of the plasmid. Product of 12α-HSDH lane [9] was sequenced and listed in (Table 6). Lanes; [2, 4, 6, 7, 10, 11, and 12] were eliminated due to the impurity.

Table 6. The nucleotide sequence of 12α-HSDH from *Clostridium scindens* ATCC 35704 (NCBI Reference Sequence: NZ_DS499706.1)

ATGGGATTTTTAACAGGTAAGACAGCCATTATCACAGGCGGGGGAAGA
GCGACATTAAGTGACGGAAGCTGTGGTTCTATTGGCTATGGGATTGCGACC
GCATACGCGAAAGAGGGGGCCAATCTGACCTTGACGGGACGTAATGTGAA
GAAACTGGAAGATGCAAAAGAAGAACTGGAGAGGCTCTACGGGATTAAAG
TGCTTGCAGTTCAGGCTGACGTAAGCGCAGGCGCTGATAATAAAGCGGTTG
TCGAACAGGTTATTAAGCAGACAGTTGAGGAGTTTGGAAGAATTGACGTGC
TGATTAATAATGCACAGGCATCTGCTTCAGGAGTTTCAATTGCCGATCACA
CGACAGAGCAGTTTGACCTGGCGATTTATTCCGGGCTGTATGCGGCTTATT
ATTATATGCAGGCGTGCTATCCGTATCTGGCAGAGGCGAAGGGAAGCGTTA
TTAACTTTGCATCAGGCGCGGGGCTGTTCGGGCATTACGGACAATGTTCTT
ACGCGGCAGCAAAAGAGGGTATCCGCGGTCTTACAAGAGTGGCTGCGACG
GAGTGGGGCAAAGACGGGATTAACGTTAATGTTGTGTGTCCTCTTGCATGG
ACAGTTCAGCTTGAGAATTTCGAGAAGGCATATCCAGATGCATTTAAAGCG
AATGTAAAGATGCCTCCGGCCGGCCACTATGGAGATGTGGAGAAGGAAAT
CGGACGCGTCTGTGTTCAGCTGGCATCTCCGGACTTCAAGTTCATGAGTGG
TGAGACTATTACGCTGGAAGGCGGAATGGGGCTTAGGCCATAA

Characterization of the Recombinant Enzyme 12α-HSDH. Since this study was chiefly dedicated to identify the conversion of 12-oxolithocholic acid to deoxycholic acid, it was important to continue this work with biochemical investigation to determine the optimal conditions for recombinant 12α-HSDH activity. Kinetic analysis was applied for the recombinant enzyme from *Clostridium scindens* ATCC 35704.

(i) Enzyme concentration and kinetic analysis. It was important to demonstrate the ideal concentration of the recombinant protein reaction. Optimal concentration of the recombinant 12α-HSDH enzyme from *C. scindens* was determined to be 8 nM for the reductive direction (12-oxolithochilic acid/ NADPH), $V_{max} = 33.69 \pm 6.32$ μmole • min^{-1} • mg^{-1}, and for NADPH $V_{max} = 24.21 \pm 2.99$ μmole • min^{-1} • mg^{-1}. For the oxidative direction (DCA/NADP) optimal concentration for the enzyme was measured to be 10 nM, $V_{max} = 116.83 \pm 5.68$ μmole • min^{-1} • mg^{-1}, and for the NADP+ $V_{max} = 95.60 \pm 7.46$ μmole • min^{-1} • mg^{-1}. Optimal concentrations for both substrates and for cofactors were determined based on Michaelis-Menten and Lineweaver-Burk plots (Figure 39-42). Data analysis was finalized using Graph Pad Prism software.

(ii) pH optimization. It was also important to vary the pH of the reaction mixture with all substrates and cofactors that regulate the active reaction toward each direction (Figure 43). pH 7.0 was determined to be the optimal for the reductive direction (12-oxolithochilic acid/ NADPH) and pH 6.5 was the optimal for the oxidative direction (DCA/NADP) (Figure 43).

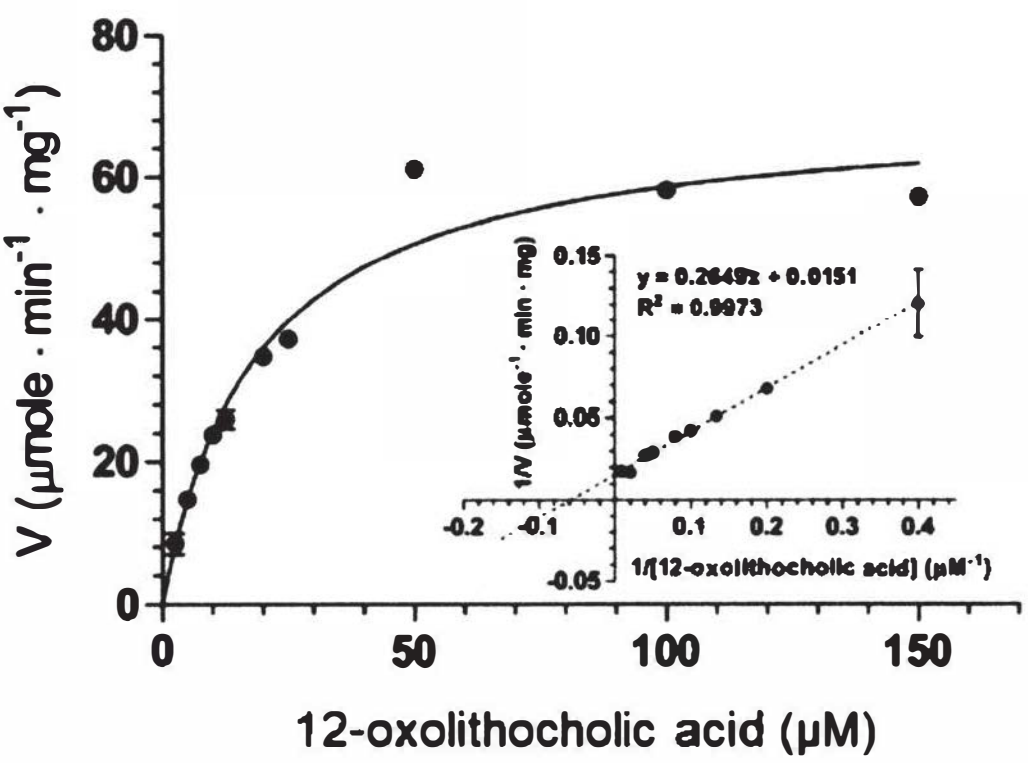


Fig 39. Biochemical characterization of recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704, and kinetic and Lineweaver-Burk plots of purified 12α-HSDH using 12-oxolithocholic acid as a substrate (1). Results were the mean of three or less replicate experiments, values represent the means ± standard deviation (SD).

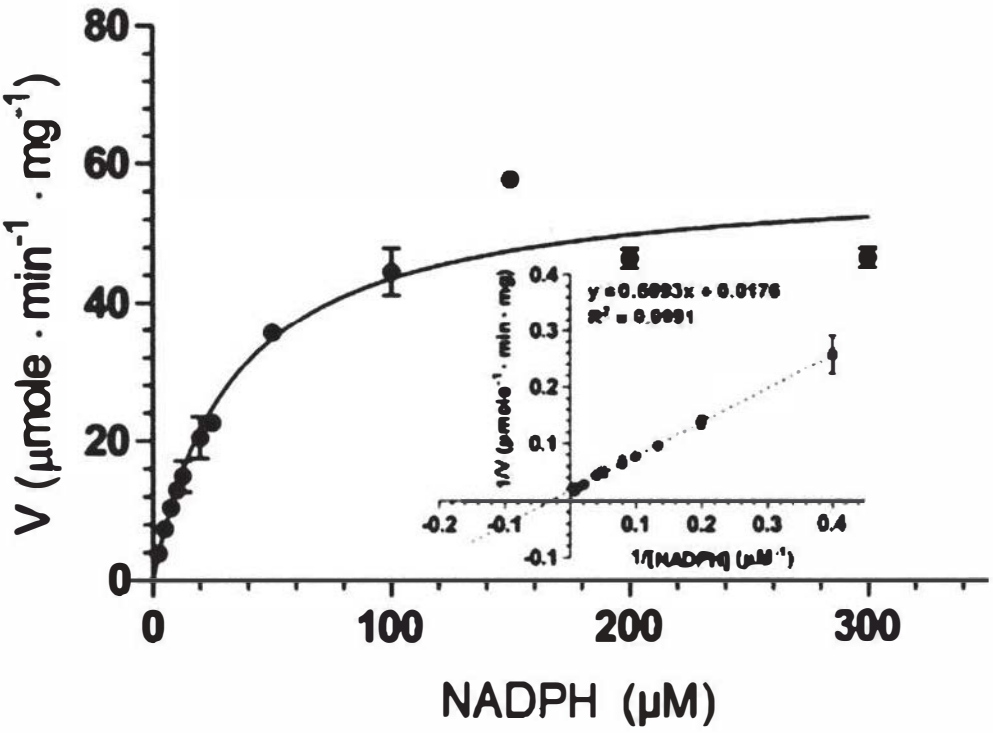


Fig 40. Biochemical characterization of recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704, and kinetic and Lineweaver-Burk plots of purified 12α-HSDH using NADPH as a cofactor (1). Results were the mean of three or less replicate experiments, values represent the means ± standard deviation (SD).

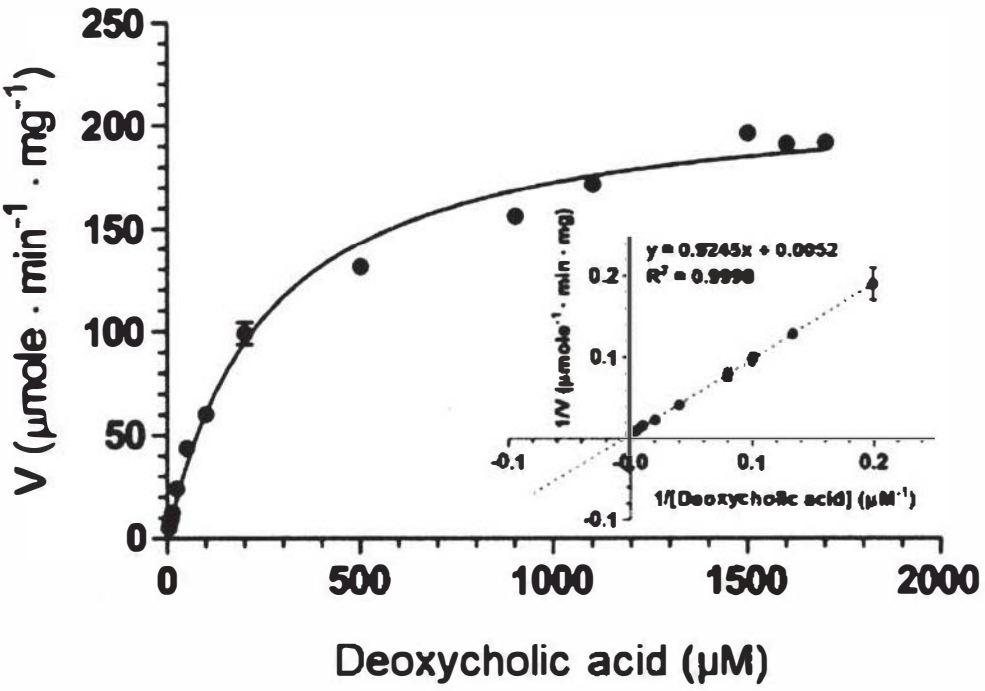


Fig 41. Biochemical characterization of recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704, and kinetic and Linweaver-Burk plots of purified 12α-HSDH using deoxycholic acid as a substrate (1). Results were the mean of three or less replicate experiments, values represent the means ± standard deviation (SD).

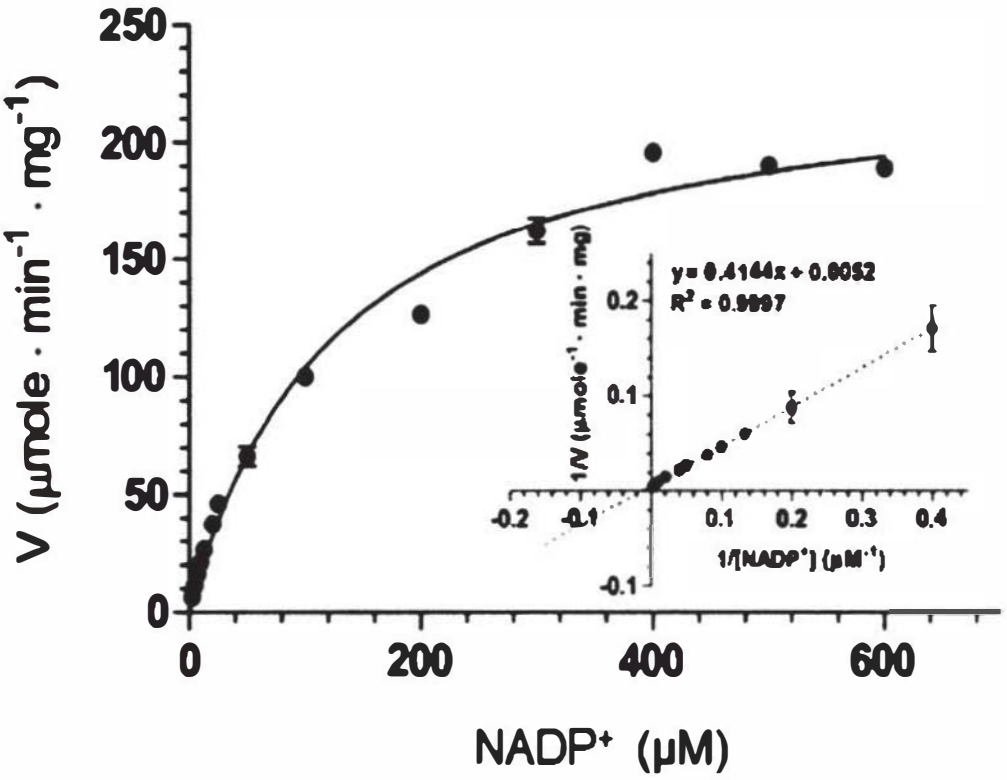


Fig 42. Biochemical characterization of recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704, and kinetic and Lineweaver-Burk plots of purified 12α-HSDH using NADP+ as a cofactor (1). Results were the mean of three or less replicate experiments, values represent the means ± standard deviation (SD).

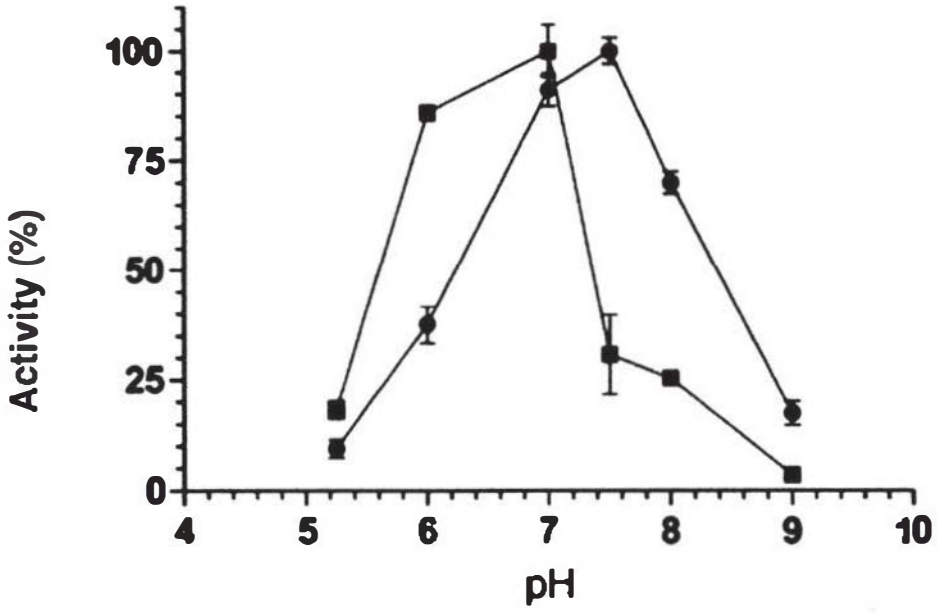


Fig 43. Biochemical characterization of recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704. Optimal pH of the reductive direction: NADPH (150 μM); recombinant enzyme (8 nM), 12-oxoLCA (50 μM), buffering agent (150 μM); and pH 7.0 sodium phosphate buffer (NaPi). Optimal pH of the oxidative direction: recombinant enzyme (10 nM); NADP+ (400 μM), DCA (1500 μM); buffering agent (150 μM); and pH 7.5 NaPi (1). Results were the mean of four or less replicate experiments, values represent the means ± standard deviation (SD). Symbols; ●, oxidation; ■, reduction.

(iii) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was applied to confirm the purity of the recombinant 12α-HSDH enzyme from *Clostridium scindens* ATCC 35704. The enzyme was purified using Streptactin™ affinity chromatography (Figure 44). The recombinant enzyme 12α-HSDH from *C. scindens* ATCC 35704 that yielded on SDS-PAGE had a deduced subunit molecular mass of 28.2 kDa, with an observed subunit mass of 27.3 0.9 kDa.

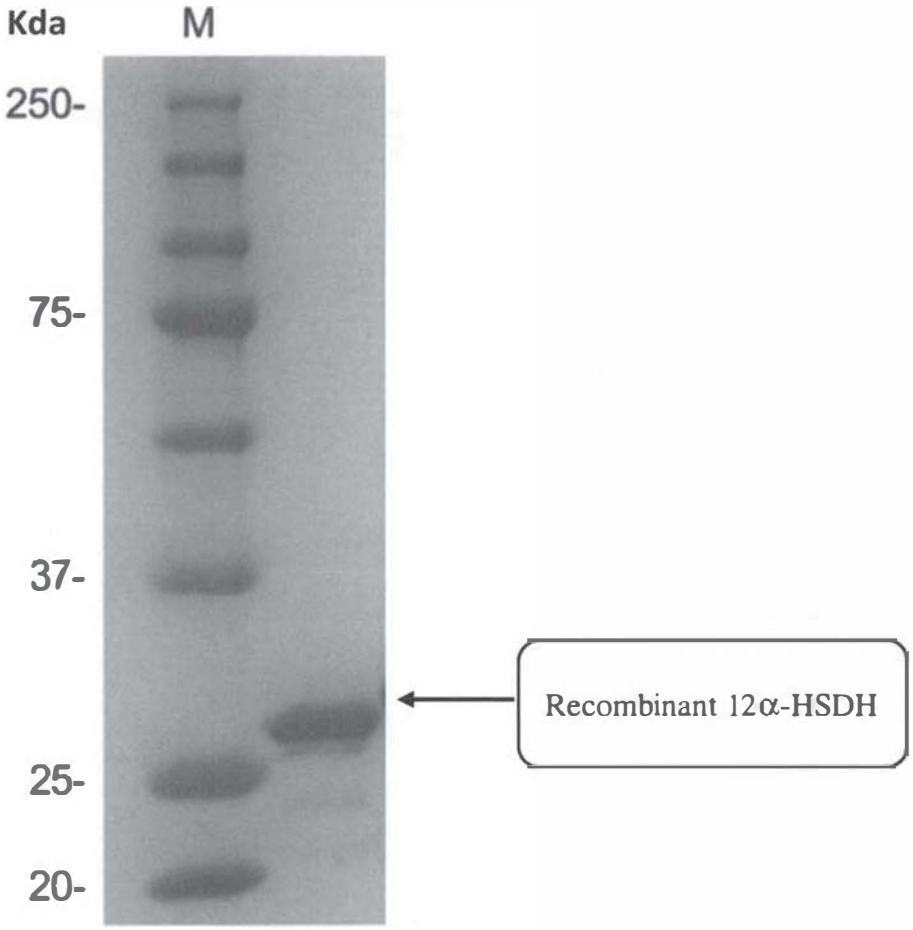


Figure 44. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of purified recombinant 12α-HSDH from *Clostridium scindens* ATCC 35704. The enzyme was purified by strep-tag affinity chromatography (1). M; molecular mass marker (Bio-Rad).

5. DISCUSSION

At the current study, the anaerobic bacterial strain *C. scindens* ATCC 35704 was found to actively convert 12-oxoLCA to DCA which is supported by the fact that this strain possess 3α-HSDH, 7α-HSDH, and 12α-HSDH (2, 4). Human gut bacteria such as *Clostridium*, *Eubacterium*, *Bifidobacterium*, and *Egghertella* are known to contribute to the occurrence of more than 20 different secondary bile acids (4). It was remarked in this study that *C. scindens* VPI 12708 was unable to convert the 12-oxoLCA to DCA, however, other studies have reported that *C. scindens* VPI 12708 actively converts the CA to DCA. Consequently, this strain was found to possess 7α-HSDH (5, 43).

When 12-oxoLCA stability was observed under different conditions, such as incubation in sterile culture media or in spent culture media, neither condition affected the nature of 12-oxoLCA nor caused 12-oxoLCA to be converted to any other form of bile acids which indicate that 12-oxoLCA does not influence by abiotic activities.

Despite the fact that non-induced cells of *C. hylemonae* were not able to produce 12-oxoLCA from either CA or 12-oxoCDCA, cells induced with the CA/12-oxoCDCA combination generated 12-oxoLCA besides other oxo-derivatives, hypothetically, the study suggests that the pre-exposure to particular bile acid may trigger the reduction and oxidation reaction to produce HSDH enzymes, but, the induction mechanism is still unexplained(1, 2). Although 12-oxoCDCA was not transformed directly to 12-oxoLCA, it was transformed to 7-oxoDCA, the latter can be converted directly to 12-oxoLCA by *C. scindens* and *C. hiranonis* (39). Only *C. scindens* ATCC 35704 and *C. hiranonis* were able to transform 7-oxoDCA to DCA and 7,12-dioxoLCA to DCA, while only *C. scindens* converted the 7-oxoDCA to CA (39). Some studies were reported that, bacterial 7α-HSDH reactions catalyzing the bioconversion of CDCA to 7-oxoLCA, that has been reported in

several intestinal bacterial species, including *C. scindens* (44, 45). However, the mechanism on how this occurs is still unknown.

Most studies were focused on the conversion of CA to DCA and CDCA to LCA by different anaerobic bacteria, but the current study showed that ~10 oxo-bile acids derivatives were involved in many transformations and in the process generated several secondary forms in different steps (39). It was clear that different strains were causing different conversions in presence of the same bile acids (Figure 45). For example, *C. scindens* ATCC 35704 was the only organism found to transform 7-oxoDCA and 7,12-dioxoLCA to CA (39). At the same time, only *C. hylemonae* was able to convert 7,12-dioxoLCA to 3-oxoCA.

No activity was visualized for 12α-HSDH when CA, 3-oxoCA, 7-oxoDCA, or DCA were added to anaerobic cultures of *C. scindens*, *C. hiranonis*, or *C. hylemonae*. However, 12α-HSDH activity was observed when cells were induced with CA prior to inoculate the cells with combination of CA and 12-oxoLCA, the CA induction may trigger/influence 3α, 7α, and 12α-HSDH enzymes to generate several derivatives, this suggested that pre-exposure to 12-oxoLCA or 12-oxoCDCA might induce HSDH activities relative to 12α-HSDH activity but it is not reported yet if the exposure to the 12-oxoLCA or 12-oxoCDCA would apply to 3α- or 7α-HSDH activities (46). However, the mechanism on how this occurs is not clearly explained.

As several substrates were tested to determine the potential impact of each compound on the transformation of 12-oxoLCA to DCA, each regulatory substrate tested in this experiment gave the same products from *C. scindens* metabolism.

Although all possible products from each transformation were able to be identified and visualized under the UV light during the TLC process, some products were hard to identify

without examining the standard of each product on the same TLC plate. It was clear that TLC might not be the optimal technique to identify the presence or absence of specific substrates or to differentiate between related substrates. Some bile acids derivatives showed similar R_f values such as 3-oxoCA and 7-oxoCA, and other similarity were found between 12-oxoLCA and 3-oxoDCA, which made identification difficult; some other intermediate bile acids were also, for the same reason, difficult to identify (39).

Even though, other techniques were used to determine the presence or the absence of bile acid such as sensitive fluorimetric assay, gas chromatography (GC), high performance thin-layer chromatography (HPLC) that used in clinical chemistry also used as a bile acids separation method, but these can only analyze either the concentration of the bile acids or part of bile acids due to the complex and similar structure of bile acids (47). Other techniques were also applied such as radioimmunoassay, bioluminescence to detect the bile acids, however, these were primarily used to detect diseases related to bile acids metabolize (47). Therefore, TLC was the suitable method to be used in this study in term of separation and differentiation the extractions based on the polarity of bile acids.

Bile acids are known to be involved in food digestion, lipid and glucose regulation, only recently have studies shown that bile acids are much more multidimensional (44, 46). Bile acids can regulate host metabolic and immune functions as well as serve as a nutrient hormone involved with signaling within the human intestine (44, 48, 49). Deoxycholate is a secondary bile salt that can induce phenotypic changes in hepatic stellate cells leading to oversecreting of pro-inflammatory cytokines that can facilitate the development of a hepatocellular carcinoma(46, 50, 51). Further studies are needed to explore the biochemical activities of the gut microbiota on bile acids and how these activities affect host physiology (9, 13). Relative to host physiology, bacterial contact with host epithelial

cells can result in the enzymatic generation of reactive oxygen species and consequent redox signaling. Interestingly, a recent report has demonstrated that some genera of human gut intestinal bacteria can induce a rapid increase of reactive oxygen species that then elicit a physiological response through the activation of epithelial NAPDH oxidase-1 (Nox1) (47). NADP-dependent 12α-HSDHs have been detected in Bifidobacterium species (Aries 1970) and *C. leptum* (37, 38) in Clostridium group P (37, 52), whereas NAD-dependent 12α-HSDH activity was reported in *Eggerthella lenta* (53) and *C. perfringens* (37). Meanwhile, 12β-HSDHs have been detected in *C. tertium, C. difficile,* and *C. paraputrificum* (38).

Up to date, *Clostridium* group P, strain C 48–50, is the only microorganism known to express HSDH activity at unusually high level, meanwhile in the absence of other HSDHs (37). 12α/β-HSDHs characterized to this point are constitutively expressed and non-inducible, with the exception of the 12β-HSDH from *C. paraputrificum,* which is induced by 12-oxo-bile acid substrates (38, 54). Generally, 12α/β-HSDHs have higher affinity for DCA than for CA and iso-CA(48). Together, the 12α-HSDH from *C. leptum* is an exception, demonstrating higher affinity for CA conjugates than for free CA (45, 55).

The reason of why *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 are involved in all of these conversions to form some toxic products from the secondary bile acids within the bacterial environment is still unknown. Hypothetically, *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053 could benefit from the toxicity of DCA to reduce the competitors for nutrients in the human intestines (39). Since 12-oxo group of 12-oxoLCA would be expected to act as an electron sink for bile acids 7α-dehydroxylating bacteria, the reduction of 12-oxo group would be favorable in an anaerobic bacterial environment (1). For many years, some

hypothesis has suggested the benefits of bile acids contribution with the human gut bacteria, the first hypothesis states that some species are able to deconjugate bile acid might be able to use the amino acid taurine as an electron acceptor (9, 10, 14). A result that advocates this hypothesis was obtained for some Clostridium species (12, 24). Other hypothesis stated that bile acids decrease the toxicity of conjugated bile acids for bacteria (19). Compared with their conjugated counterparts, deconjugated bile acids have reduced solubility and diminished detergent activity and may, therefore, be less toxic to bacteria in the intestine (6). However, further studies are still needed to comprehend the importance of this conversion clearly.

In steroid metabolism, HSDH enzymes play an important role for gut bacteria. *C. scindens* ATCC 35704 was reported to express 3α-HSDH, 7α-HSDH, 20α-HSDH and steroid-17, 20-desmolase as part of the complex bile acid 7α-dehydroxylation pathway (19). The 7α-HSDH may serve two functions; i) convert 7-oxo-bile acids generated by other gut bacteria to 7α-hydroxy bile acids (20) and ii) regulate the flux of bile acids into the 7α-dehydroxylation pathway by reversible oxidation-reduction of the 7α-hydroxy group (34). Gut bacteria such as *Clostridium baratii*, *B. producta* , and *Ruminococcus gnavus* are also capable of forming 7β-hydroxy bile acids, including *C. scindens* and other bile acid 7-dehydroxylating bacteria circumvent this need by expressing a 7β-dehydratase (21, 22, 23). Interestingly, epimerization of the 3α-hydroxy group to 3β-hydroxy "iso-bile acids" prevents 7α-dehydroxylation (J. M. Ridlon and P. B. Hylemon, unpublished observations). *C. scindens* lacks 3β-HSDH, and the first enzymatic step following bile acid CoA-ligation is oxidation of the 3α-hydroxy group (20). Even though this study shows that *C. scindens* possess 12α-HSDH, it is not reported if *C. scindens* possess 12β-HSDH (1). 12β-HSDHs have been detected in different members of the Firmicutes, however, up

to date no isolate from *Clostridium* has been found to possess both 12α- and 12β-HSDHs (57). It might be an important to understand the hypothesis of the 12-oxoLCA activity in the host environment, and how that can impact the metabolism of the host secretion and microorganism as well. Further studies are needed to fully comprehend the hypothesis of the interaction between gut microbiota, host secretion, and bile acids and to determine the impact of these factors on steroidal bile acids transformation along with the HSDH enzymes activity.

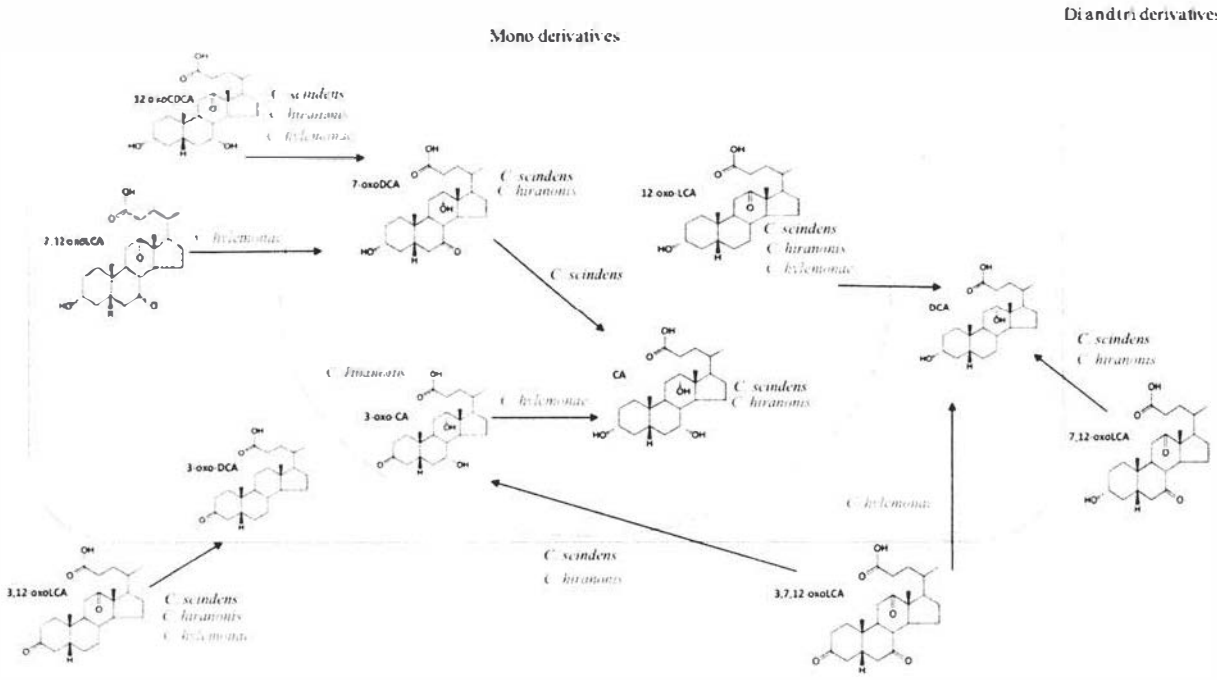


Fig 45. Effect on structure of bile acids transformation; all mono-oxo bile acids derivatives that generate DCA were indicated inside the green circle, other mono-oxo bile acids derivatives were indicated inside the orang box, and all di/ tri-oxo bile acids derivatives were indicated outside the orange box. Bile acids biotransformation were generated by *C. scindens* ATCC 35704, *C. hiranonis* DSM 13275, and *C. hylemonae* DSM 15053.

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