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Article

Bile Acid Analogs with Anti-Germination Activities for Prophylaxis of *Clostridioides difficile* Infection Alter Bile Acid Homeostasis in the Enterohepatic Cycle

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Abstract

We previously reported that two bile acid (BA) analogs CamSA and CA-Quin demonstrate potent anti-germination activity against *Clostridioides difficile* (*C. difficile*) spores, protecting rodents from *C. difficile* infections. Here we further evaluated the impact of these analogs on the hepatic transcriptome and BA homeostasis *in vivo* by focusing BA profiles on the liver, feces, and chyme as well as hepatic transcriptome after a 7-day treatment. The two compounds demonstrated similar impact on BA profiles among the three samples, with significantly increased BA excretion in feces. This change is aligned with significantly altered expression of genes involved in BA homeostasis in both liver and gut tissues. Also, both compounds increased levels of unconjugated BAs in the feces, indicating an elevated activity of gut microbiota (GM). Notably, fecal levels of anti-*C. difficile* germination chenodeoxycholate and pro-germination taurocholate are significantly increased and decreased by the treatments, respectively. While hepatic transcriptome did not show significant toxicity, altered genes are enriched in pathways associated with GM activity and lipid metabolism. Overall, our study suggests that *in vivo* CamSA and CA-Quin treatment demonstrated minimal hepatotoxicity but significantly altered BA homeostasis and potentially favorable improvement for GM profiles that together inhibit *C. difficile* germination.

Keywords: *C. difficile*; bile acid; enterohepatic circulation; gut microbiota; transcriptome

1. Introduction

Clostridioides difficile, (*C. difficile*), is a species of bacteria that is the world's leading cause of antibiotic-associated diarrhea [1]. *C. difficile* infection (CDI) is mainly spread in health-care settings; however, community spread of CDI is increasing [1-3]. About 500,000 people are infected with *C. difficile* every year, with an almost 6% mortality rate [1-3].

CDI symptoms range from mild diarrhea to deadly colitis with the variable symptomatology being attributed to individual susceptibility and complex antibiotic regimes [4]. Primary CDI is mainly treated with vancomycin or fidaxomicin, but up to 20% of patients experience relapses [5].

C. difficile is spread by spores, which are dormant and resistant structures that allow *C. difficile* to survive harsh conditions for long periods. *C. difficile* spores germinate in the colon of susceptible

patients, a process required to initiate CDI [1-3]. Once spores germinate in the antibiotic-altered gut of patients, they generate toxin-producing vegetative cells [4]. Given the risk of antibiotic resistance and treatment-induced dysbiosis of the gut microbiota, there is an urgent need to discover new therapeutics to treat CDI.

The germination of *C. difficile* spores is mediated by several factors including the composition of bile acids in the gut. Taurocholate (TCA), a conjugated primary bile acid, facilitates germination while chenodeoxycholate (CDCA), an unconjugated primary bile acid, inhibits it [6, 7]. Bile acids are synthesized in the liver and secreted into the intestines where their primary function is to emulsify and facilitate the absorption of fats and vitamins [8, 9]. Bile acids synthesized endogenously in the liver are classified as primary bile acids. In humans, these are cholic acid (CA) and chenodeoxycholic acid (CDCA) [8, 9]. Mice have 3 additional endogenous bile acids: ursodeoxycholic acid (UDCA), alpha-muricholic acid (α MCA), and beta-muricholic acid (β MCA) [8]. Once primary bile acids are synthesized in hepatocytes, either taurine (T) or glycine (G) are attached to the carboxylic acid to form conjugated bile acids which require specific transporters to move across the canalicular membranes [8, 9]. Once bile acids reach the intestinal ileal lumen, bacteria can deconjugate and dehydroxylate them into secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA), respectively [8, 9]. By the time they reach the end of the ileum, about 95% of the bile acids are reabsorbed by enterocytes and transported back to the liver through the enterohepatic circulation (EHC), which continuously recycles bile acids between the intestines and the liver [8, 9]. Secondary bile acids are absorbed from the intestine and reconstituted in the liver before re-entering the EHC [9].

Since bile-acid-mediated spore germination is required for CDI, we expect that anti-germination therapy will prevent CDI and its relapse [10]. We have tested synthetic bile salt (SBS) libraries and found analogs that inhibit *C. difficile* spore germination [11-14]. More importantly, SBS anti-germinants also prevented CDI in rodents [15-18] without any overt toxicity seen after a 30-day treatment. More specifically, we found that cholates like cholan-24-amide containing *m*-sulfanillic acid (CamSA) [19, 20] and quinazoline cholate analog (CA-Quin), inhibit *C. difficile* spore germination at micromolar concentrations even in the presence of saturating millimolar levels of the germinant taurocholate germinant is present. Given that CamSA was the first CDI prophylactic discovered in our group, it has been extensively characterized both *in vitro* and *in vivo* [15-18, 21-23].

One surprising finding was that a single dose of CamSA was sufficient to protect mice from CDI. To study this intriguing pattern, we examined the pharmacokinetics of CamSA [21]. Our data suggests that CamSA undergoes EHC. We hypothesize that the cycling of CamSA between the liver and intestines serves as a slow-release mechanism that allows CamSA to be retained in the gastrointestinal tract for days. This model explains how a single CamSA dose can provide multi-day protection against murine CDI [18]. Although CA-Quin also protects mice from CDI, we have not determined whether it enters the EHC.

Given the involvement of CamSA in the EHC and the fact that these agents are bile salt analogs, it is unclear whether they affect regular circulation or metabolism of natural bile acids. It also remains to be further assessed whether CamSA and CA-Quin have toxicological impacts on the liver. In this study, we treated mice with CamSA, CA-Quin, or vehicle control for 7 days. The liver histology, transcriptome, and bile acids profiles of the liver, ileum, chyme, and feces samples were analyzed and compared. In conjunction with previous studies, the present study indicates that the two bile acid analogs are safe and alter bile acids profile in a manner benefiting *C. difficile* inhibition. These findings warrant further investigation into their potential for the clinical treatment of CDI.

2. Materials and Methods

2.1. Materials

All materials used in the assays of this study were summarized in supplementary Table S1.

2.2. Synthesis of CamSA and CA-Quin:

The synthesis of CamSA[19] and CA-Quin[24] were done according to previously published methods by the Dr. Firestine lab at Wayne State University.

2.3. Animals

Animal protocols were performed in accordance with the Guide for Care and Use of Laboratory Animals outlined by the National Institutes of Health. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Las Vegas (Permit Number: R0914-297, approval date: June 20th, 2025). Weaned male C57BL/6J mice were obtained from Jackson Labs, Jax west (Bar Harbor, ME). Mice were housed in groups of five mice per cage at the University of Nevada, Las Vegas animal care facility. Upon arrival at the facility, mice were quarantined and allowed to acclimate for at least one week prior to experimentation. Animals were 5-8 weeks old and were between 20-25 g. Tissue samples were collected after euthanasia. Therefore, no anesthesia was utilized. Drugs were administered by oral gavage and dissections were conducted after euthanasia.

2.4. Anti-germinant treatment and organ harvest

Male mice (n=4 per group) were be treated with a once daily dose of DMSO, 50 mg/kg CamSA or 50 mg/kg CA-Quin for 7 consecutive days. Feces were collected from individual animals after 3 days and 7 days of treatment. On day 7, all animals were euthanized with CO₂ and organs were harvested as follows.

Livers from each animal were harvested and weighted. A liver slice (not wider than 0.5 cm) was stored in cold RNeasy Protect Tissue Reagent. A second part (200-300 mg) was chopped, placed into aluminum foil, and flash frozen. Foils were then transferred into pre-cooled Eppendorf tubes and stored at -80 C.

A 15-cm segment of the ileum proximal to the ileocecal valve was cut from each animal and flushed with extraction buffer (0.1 M Tris-buffered saline with 0.3% bovine serum albumin, 0.01% sodium azide, and 0.002% Tween). The resulting intestinal content suspensions (chyme) were flash frozen and stored at -80 C. The cleaned ileums were sliced lengthwise and cut into equal sized pieces. One piece was stored in cold RNeasy Protect Tissue Reagent. Three other ileum pieces were put into foil, as above.

RNA Extraction: Frozen liver samples stored in Qiagen RNeasy Protect Tissue Reagent were homogenized and RNA was extracted utilizing the Qiagen RNeasy Kit by following the manufacturer's protocol. Additional flash frozen liver and ileum samples were homogenized in ThermoFisher Scientific TriZol and extracted by following manufacturer's protocol.

Sequencing: RNA sequencing was performed by the Wayne State Genomics Core. Briefly, total RNA was extracted from frozen liver and ileum samples using TRIzol reagent (Thermo Fisher Scientific) followed by purification steps as detailed by the manufacturer. RNA purity and quantity were assessed via spectrophotometer (NanoDrop 8000, Thermo-Fisher Scientific). Single end RNA sequencing was performed by the Wayne State University Genome Sciences Core. The mRNA-seq library was prepared using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen). Libraries were assessed by the High Sensitivity D1000 (HS D1000) ScreenTape Assay (Agilent). Samples were sequenced on the NovaSeq system (Illumina).

2.5. RT-PCR and qPCR

Extracted RNA from frozen tissue was utilized for RT-PCR which was performed utilizing ThermoFisher High-Capacity cDNA Reverse-Transcription kit. qPCR was conducted utilizing the ThermoFisher PowerUp SYBR Green Master Mix for qPCR for the liver samples and ileum samples (in technical triplicates) in a QuantStudio qPCR machine utilizing manufacturer's protocol. Primer sequences are included in supplementary Table S2.

2.6. LC-MS of bile species

LCMS was performed by the Zhou lab at the Medical College of Virginia. Serum and liver bile acids were extracted and quantified by LC-MS/MS as previously described [25, 26]. The following bile acids were measured (in nmol/g): 12-keto-lithocholic acid (12-keto-LCA), 3-keto-7 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (3-keto,7 α ,12 α (OH)₂), 3-keto-lithocholic acid (3-keto-LCA), 7-keto-deoxycholic acid (7-keto-DCA), 7-keto-lithocholic acid (7-keto-LCA), allo-isolithocholic acid (allo-isoLCA), alpha-muricholic acid (α -MCA), beta-muricholic acid (β -MCA), 7 α -hydroxy-4-cholesten-3-one (C4), cholic acid (CA), cholic acid-3-sulfate (CA-3-S), cholic acid-7-sulfate (CA-7-S), chenodeoxycholic acid (CDCA), chenodeoxycholic acid-3-sulfate (CDCA-3-S), deoxycholic acid (DCA), deoxycholic acid-3-sulfate (DCA-3-S), glyco-beta-muricholic acid (G β -MCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycohyocholic acid (GHCA), glycohyodeoxycholic acid (GHDCA), glycolithocholic acid (GLCA), glyoursodeoxycholic acid (GUDCA), hyocholic acid (HCA), hyodeoxycholic acid (HDCA), isodeoxycholic acid (isoDCA), isolithocholic acid (isoLCA), lithocholic acid (LCA), lithocholic acid-3-sulfate (LCA-3-S), murideoxycholic acid (MDCA), tauro-alpha-muricholic acid (T α -MCA), tauro-beta-muricholic acid (T β -MCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurohyodeoxycholic acid (THDCA), tauroolithocholic acid (TLCA), taoursodeoxycholic acid (TUDCA), tauro-omega-muricholic acid (T ω -MCA), ursodeoxycholic acid (UDCA), ursodeoxycholic acid-3-sulfate (UDCA-3-S), omega-muricholic acid (ω -MCA). Samples were filtered (0.2 μ m PTFE) and analyzed using a Shimadzu LCMS-8600 system.

2.7. Differential gene expression analysis

Sequencing data from the WSU Genomics Core was analyzed for quality by FastQC[27] and then trimmed by Trimmomatic [28] and rechecked for quality. RNA reads were mapped to the mouse genome utilizing Hisat2[29]. Gene counts were collected utilizing HTSeq[30, 31] and then the data was analyzed in R[32] utilizing RStudio[33]. In R, DESeq2[34] was utilized to perform differential gene expression and obtain normalized counts. Ggplot2[35] was utilized to generate the principal component analysis and volcano plots. Venn diagram plotted using ggvenn[36]. Complex heatmap[37, 38] was utilized for the heatmaps. Additional packages utilized for intermediary steps necessary for analysis, data handling, data annotation, and figure adjustments include: KEGGREST, biomaRt[39, 40], dplyr[41], ggrepel[42], AnnotationDbi[43], org.Mm.eg.db[44], ggfortify[45, 46], patchwork[47], and openxlsx[48].

2.8. Enrichment analysis

For enrichment analysis, genes were filtered based off a p value of 0.05. These genes were then run in Metascape [49] for each treatment and the GO, KEGG, and Reactome databases were selected for the custom analysis. The enrichment analysis parameters were a p value of less than 0.01 and the minimal overlap was 3 genes.

2.9. Statistical analysis

Bile acids concentration data were log₂ transformed. For bile acid ratio, data were log₂ transformed before calculating the ratio. Percent composition was calculated by dividing the concentration of individual species by the total concentration quantified in a sample. 2^{- $\Delta\Delta$ CT} was quantified utilizing Microsoft Excel (Version 16.94 (25020927), 2025 Microsoft) before transferring data for statistical analysis. One way ANOVA and data visualization were conducted utilizing GraphPad Prism (Version 10.4.1(532), GraphPad Software, LLC). No corrections were applied to p values for t tests. (**** p<0.0001, *** p<0.001, ** p<0.01, *p<0.05). Detailed statistics include mean, standard deviation, and statistical significance of bile salt level comparison are available upon request.

3. Results

To investigate the effects of the bile acid analogs on the EHC, mice were treated with either DMSO, CamSA, or CA-Quin for seven consecutive days. Bile acid species (n=43) of the liver (n=4) and chyme (n=4) were profiled on day 7 utilizing LC-MS/MS. Additionally, feces from days 3 (n=4) and 7 (CamSA n=3, CA-Quin n=4, and DMSO n=4) of treatment were also analyzed. A total of 42 bile acids were quantified of those there were 5 primary bile acids, 9 conjugated primary bile acids, 20 secondary bile acids, and 8 conjugated secondary bile acids. The categorization of the bile acids detected by LC-MS is indicated in the supplementary Table S3.

In addition to the 42 bile acids, 1 bile acid precursor was also quantified, 7- α -hydroxy-4-cholesten-3-one (C4). The bile acids were divided according to where or how they are made. Primary bile acids are produced in the liver and then conjugated with either taurine or glycine to form the conjugated primary bile acids. The secondary bile acids are derived from primary bile acids by bacterial biotransformation. For analysis of the bile acids, only bile salts that were quantified in at least 3 samples of one treatment group were included for analysis. In the supplementary Table S4, we have indicated how many bile acids were at quantifiable levels for each treatment.

3.1. Overall alterations of bile acids by CamSA and CA-Quin in the liver, chyme, and fecal samples

A principal component analysis (PCA) plot of the BA profile data shows a trend of separation between the two treatments groups and the DMSO control group in the liver and feces, but not in chyme, while the overall variability between the two treatment groups is less noticeable (Figure 1).

Figure 1

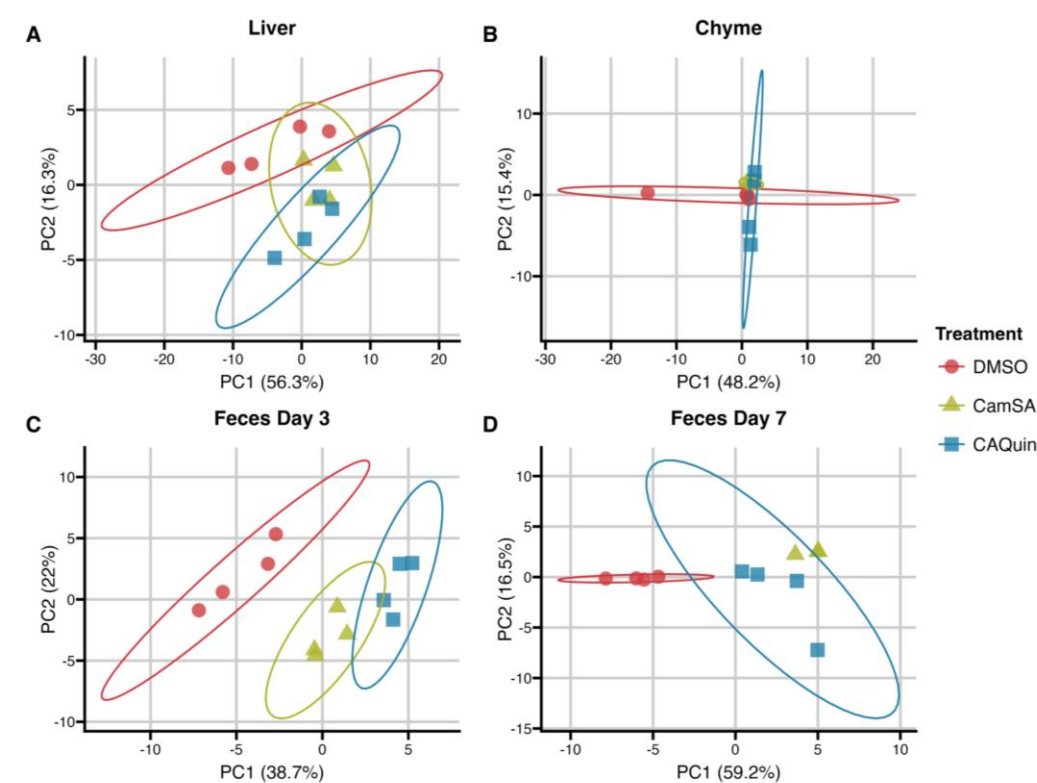


Figure 1. PCA Plots of the quantified bile species within each sample. The raw bile acid concentration was utilized to generate these PCA plots.

Neither CamSA nor CA-Quin altered the total level of bile acids compared to the DMSO-treated group among the liver, chyme, and the feces collected on day 3. However, total BA content significantly increases in the feces collected on day 7 of both treatment groups (Figure 2A). When

comparing the ratio of total unconjugated and conjugated BAs, both treatment groups demonstrated a significant trend towards lower ratios of unconjugated:conjugated BAs in the liver whereas higher ratios of unconjugated:conjugated BAs in the feces, compared to the control group (Figure 2B).

Figure 2

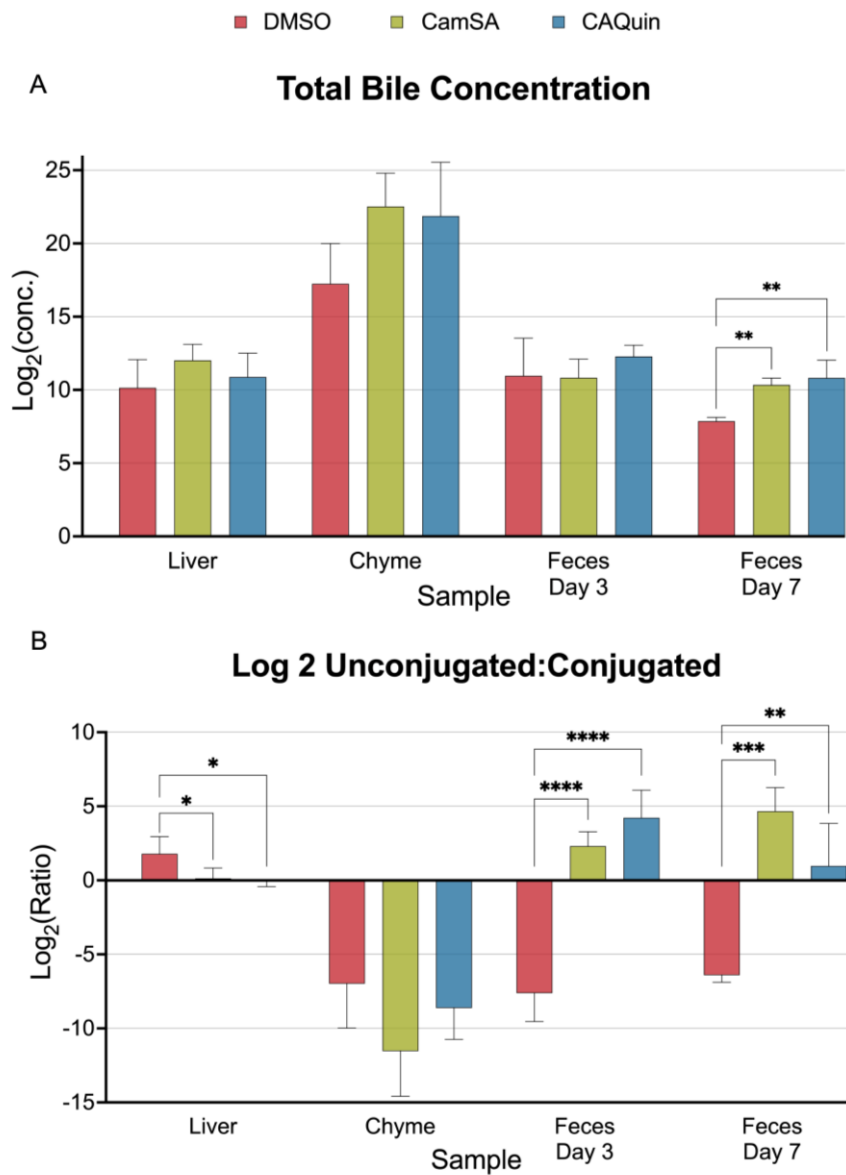


Figure 2. Overall statistics of bile acid quantifications for the 4 tissue samples. A) The total concentration of bile acids quantified in each sample was summed up and log2 transformed. B) Ratio between the total level of unconjugated bile acids and total level of conjugated bile acids. Statistics was based on the post hoc Tukey pairwise test under One-way ANOVA after the ratio was log2 transformed. (**** <0.0001, *** <0.001, ** <0.01, * <0.05).

When analyzing the changes of BA categories (primary, primary conjugated, secondary, and secondary conjugated), CamSA or CA-Quin treatments did not significantly change the total BA levels of each category in the liver or chyme (Figure 3A-B). In contrast, both unconjugated primary and secondary BAs are significantly increased in the feces (both day 3 and day 7) of both treatment groups compared to the control group (Figure 3C-D). In the feces collected on day 7, the conjugated primary BAs are significantly underrepresented in the CamSA-treated group and overrepresented in the CA-Quin treated group (Figure 3D).

Figure 3

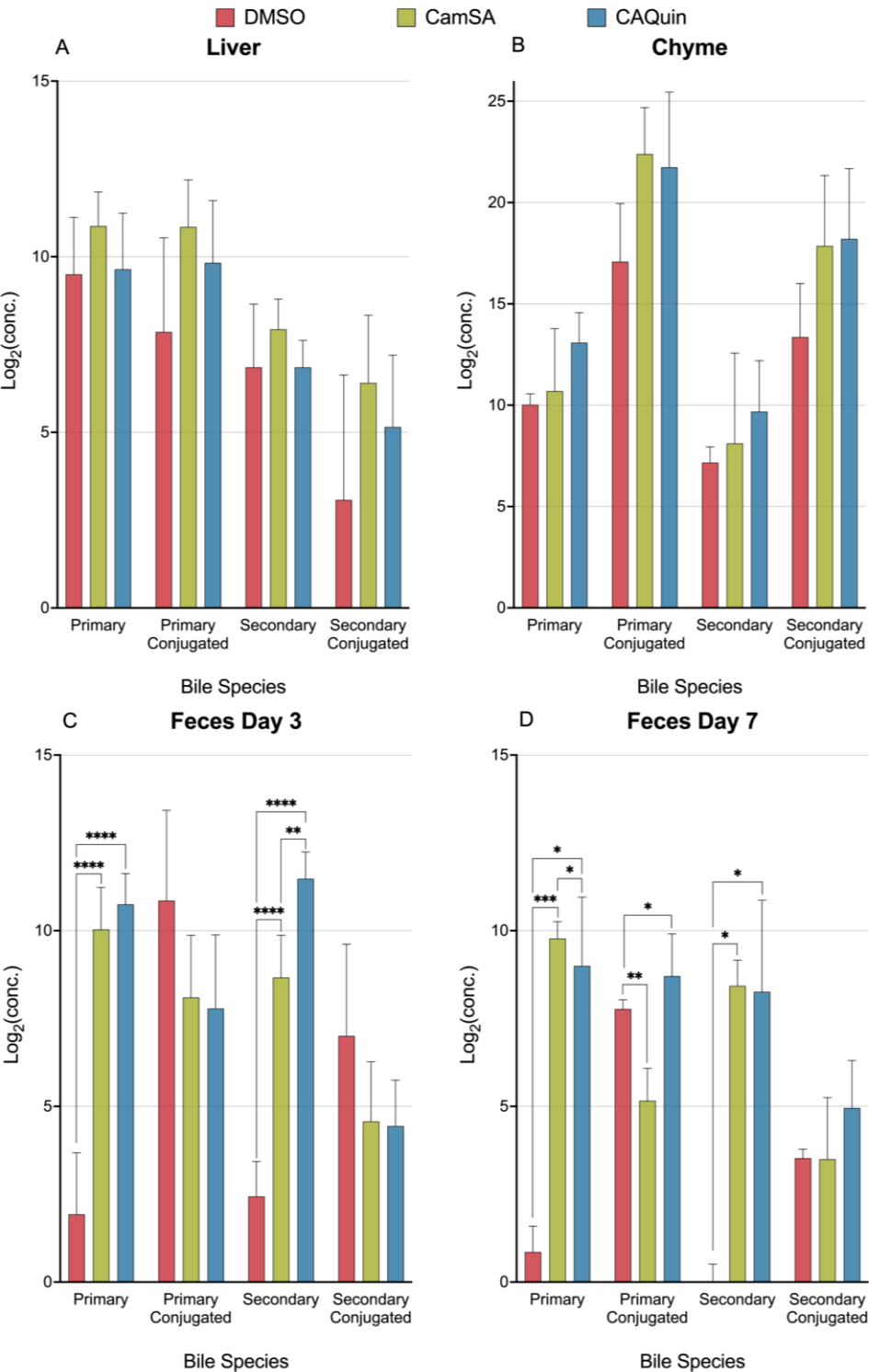


Figure 3. Levels of the total bile acids in four categories for each tissue sample. Statistics was based on the post hoc Tukey pair-wise test under One-way ANOVA after the ratio was log2 transformed. (**** <0.0001, *** <0.001, ** <0.01, * <0.05).

3.1.1. Alternation of individual BA levels by CamSA and CA-Quin treatment

Analysis of individual bile acid concentration changes showed that the major significant changes in the level of BAs are in the fecal samples of animals treated with the two compounds, with fewer significant changes observed in the liver or chyme samples (Figure 4). Nearly all five primary BAs

demonstrated an increased level in the feces (both day 3 and day 7) of both CamSA and CA-Quin-treated mice, compared to the DMSO-treated control group. In contrast, only CDCA increases in the liver of CamSA-treated mice, while both CA and CDCA increased in the chyme of CA-Quin-treated mice (Figure 4).

Figure 4

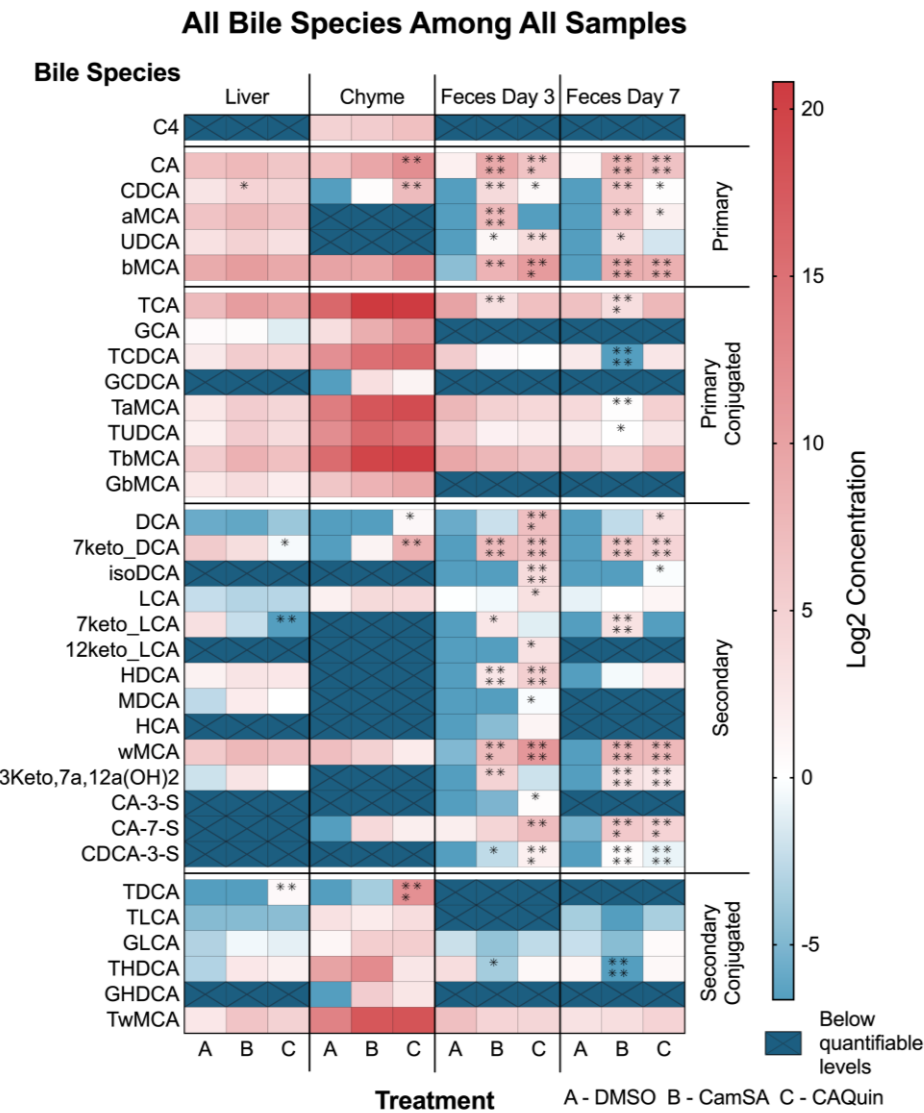


Figure 4. Level of each bile acid within the four tissue samples. Statistics was based on the post hoc Tukey pair-wise test under One-way ANOVA after the ratio was log2 transformed. (**** <0.0001, *** <0.001, ** <0.01, * <0.05). Heatmap was generated based on the log2 transformed data. Hashed cells denote bile salts species undetectable in the sample.

No significant change was observed for conjugated BAs in the liver tissue for any of the treatment groups compared with the DMSO group. In feces samples, all conjugated BAs showed some decrease in both CamSA and CA-Quin-treated mice on day 3, but only TCA showed a statistical significance in the CamSA group. On day 7, feces showed that more species (TCA, TCDCA, TaMCA, and TUDCA) had a statistical significance decrease in the CamSA-treated (but not the CA-Quin-treated group) mice (Figure 4).

Among the 14 secondary BAs that were examined, 7-keto-DCA and 7-keto-LCA levels showed a significant decrease in the liver tissue of mice treated with CA-Quin. The same group also demonstrated a significantly increased level of DCA and 7-keto-DCA in the chyme. In contrast, most

of the detectable secondary BAs in feces showed a statistical increase on day 3 and day 7 in both the CamSA and CA-Quin-treated group (Figure 4).

Only a few conjugated secondary BAs demonstrated statistically significant changes. TDCA levels in the liver and chyme of the CA-Quin group are significantly increased, while THDCA levels significantly decreased in day 3 fecal samples of the CamSA group and in day 7 fecal samples of the CA-Quin group.

Besides the absolute concentration as demonstrated above, we also further examine the relative composition of individual BAs among the samples (Supplementary Figure S1). While the pattern of relative level of BAs largely resembles that of the absolute BA concentration shown in Fig 4, several BAs demonstrated stronger association with the two treatments. For example, compared to the control group, the relative TCA level in both treatment groups is significantly increased and decreased in the liver and fecal samples, respectively (Supplementary Figure S1).

3.2. Liver Transcriptomics

To examine the impact of CamSA and CA-Quin treatments on the murine liver transcriptome, RNA sequencing was performed [DMSO (n=8), CamSA (n=8), CA-Quin (n=7)]. PCA plots showed overall minimal significant changes in the hepatic transcriptome program due to CamSA or CA-Quin treatments (Figure 5A).

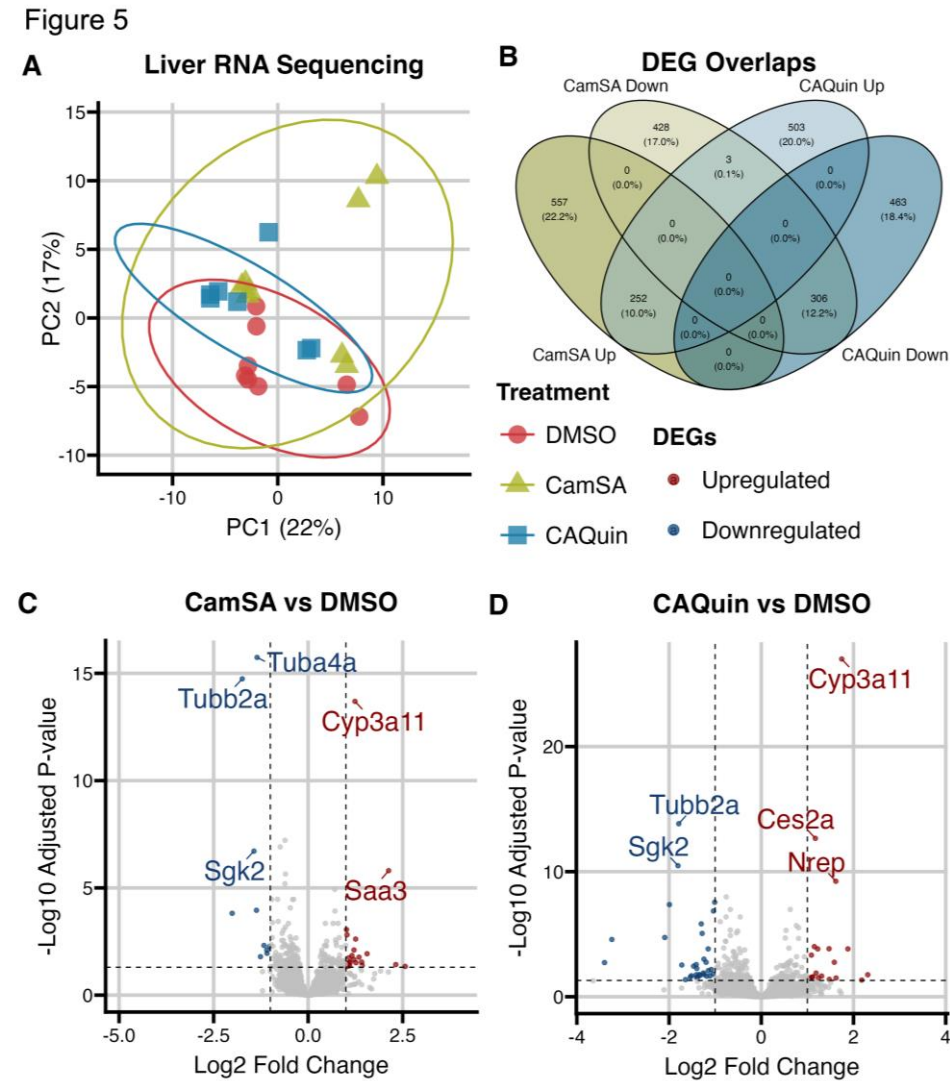


Figure 5. Hepatic transcriptomic analyses. A) PCA plot of the overall hepatic transcriptomic data of the three groups. B) Venn diagram showing the number of up- and down-regulated genes as well as shared genes in each

group. Genes with nominal $p < 0.05$ were utilized for plotting the Venn diagram. “Down” indicates the downregulated genes and “Up” indicates the upregulated genes. The corresponding percentage is out of the total number of genes with a $p < 0.05$. C and D) Volcano plots of the DEGs for CamSA and CA-Quin treatment groups, respectively. Highlighted genes had a $-\log_{10}(\text{adjusted } p) < 0.05$ and a $\log_2$ fold change ± 1.5 . The highlighted genes in blue were downregulated while the genes in red were upregulated as compared to the control group.

3.2.1. Genes and transcriptome patterns associated with drug treatments

While there was minimal difference in overall toxicogenomic effects due to anti-germinant treatments, we further examined the subset of genes that demonstrated statistically significance (unadjusted $p < 0.05$) changes in their transcription levels between each of the treatment group and the control group. A total of 809 and 758 genes are significantly upregulated in the CamSA and CA-Quin-treated groups compared to the control. Among these, 252 (>30% of significantly upregulated differentially expressed genes (DEGs) in either treatment group vs the control group) genes are shared between the two treatment groups. Similarly, a total of 737 and 769 genes, respectively, are significantly downregulated in the CamSA and CA-Quin-treated groups compared to the control. Among these, 306 (~40% of significantly downregulated DEGs) genes are shared between the two treatment groups. Only 3 genes that are downregulated in the CamSA group were upregulated in the CA-Quin treatment, while no genes upregulated in the CamSA group are downregulated in the CA-Quin treatment (Figure 5B).

Volcano plots (Figure 5C and 5D) showed that in the CamSA-treatment the 5 most differentially expressed genes (adjusted $p < 0.05$, $|\log_2 \text{fold change}| > 1.5$) were *Tuba4a*, *Tubb2a*, *Cyp3a11*, *Sgk2*, and *Saa3*. Of those, *Tuba4a*, *Tubb2a*, and *Sgk2* were downregulated while *Cyp3a11* and *Saa3* were upregulated. In the CA-Quin-treated group, the 5 most differentially expressed genes were *Cyp3a11*, *Tubb2a*, *Ces2a*, *Sgk2*, and *Nrep*. Of those, *Tubb2a* and *Sgk2* were downregulated while *Cyp3a11*, *Ces2a*, and *Nrep* were upregulated.

To further explore insights into the potential pharmacological effects related to the two treatments, we performed pathway enrichment analyses by focusing on all genes whose expression was significantly (unadjusted $p < 0.05$) altered as compared to the control group, regardless of their fold change. The Metascape [49] enrichment analysis (Fig. 6) showed the top gene ontology (GO) pathways enriched in the CamSA-related DEGs include *monocarboxylic acid metabolism*, *neutrophil degranulation*, and *regulation of response to biotic stimulus* (Figure 6A). The top GO pathways enriched in CA-Quin-related DEGs are *monocarboxylic acid metabolism*, *small molecule biosynthetic process*, and *nucleobase-containing small molecule metabolic process* (Figure 6B). Shared pathways between the two groups include *monocarboxylic acid metabolism*, *protein processing in the endoplasmic reticulum*, and *small molecule catabolic process* (Figure 6C).

Figure 6

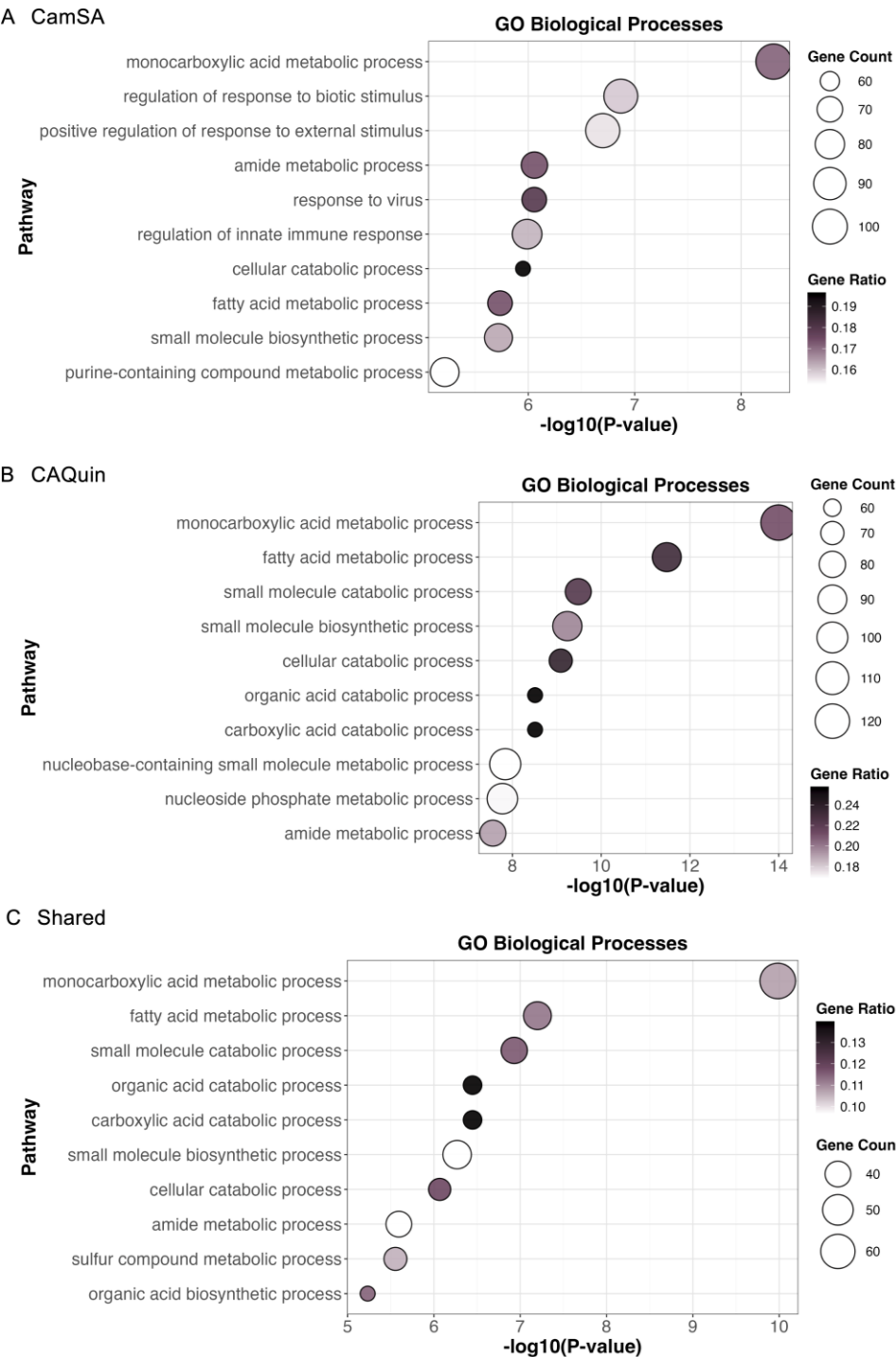


Figure 6. Enrichment analysis of transcriptomic hepatotoxicity data. All genes with a p value less than 0.05 for the respective treatment group from the DEG analysis utilized in previous figures were utilized. Analysis was done utilizing Metascape. A custom analysis utilizing GO, KEGG, and Reactome pathways was utilized with a p value less than 0.01 and minimum overlap of 3 genes. Figures are generated by the Metascape website analysis tool. A and B) Respective genes for each treatment with a p value less than 0.05 were utilized to generate the enrichment analysis. C) Genes in both treatments with a p value less than 0.05 were utilized to generate the enrichment analysis.

In addition to GO pathways, the KEGG and Reactome were investigated during the Metascape enrichment analysis as seen in supplementary Figures S2-4. From the CamSA-related DEGs, the

protein processing endoplasmic reticulum, peroxisome, and prion disease KEGG pathways were the most enriched. The Reactome pathways most enriched from the CamSA-related DEGs were *neutrophil degranulation*, *antigen processing-cross presentation*, and *metabolism of lipids*. The top KEGG pathways enriched from CA-Quin-related DEGs were protein processing in *endoplasmic reticulum*, *peroxisome*, and *biosynthesis of cofactors*. The Reactome pathways most enriched were *metabolism of lipids*, *fatty acid metabolism*, and *protein localization*. When looking at the shared DEGs, the most enriched KEGG pathways include *protein processing in endoplasmic reticulum*, *peroxisome*, and *prion disease*. The most enriched Reactome pathways were *metabolism of amino acids and derivatives*, *metabolism of lipids*, and *fatty acid metabolism*.

3.3. Impact of drug treatment on bile acid metabolism pathway in the liver

Both CamSA and CA-Quin are CA analogs and CamSA has been demonstrated to enter the EHC. Meanwhile, both treatments led to significantly altered BA profiles as noted above. Therefore, we conducted a targeted analysis on the expression of key genes involved in BA homeostasis. A heatmap of the genes involved in the pathways for primary bile acid synthesis and bile secretion (extracted from KEGG [50-52]) showed several genes that demonstrated a statistically significant change in the treatment groups compared to the control group (Figure 7).

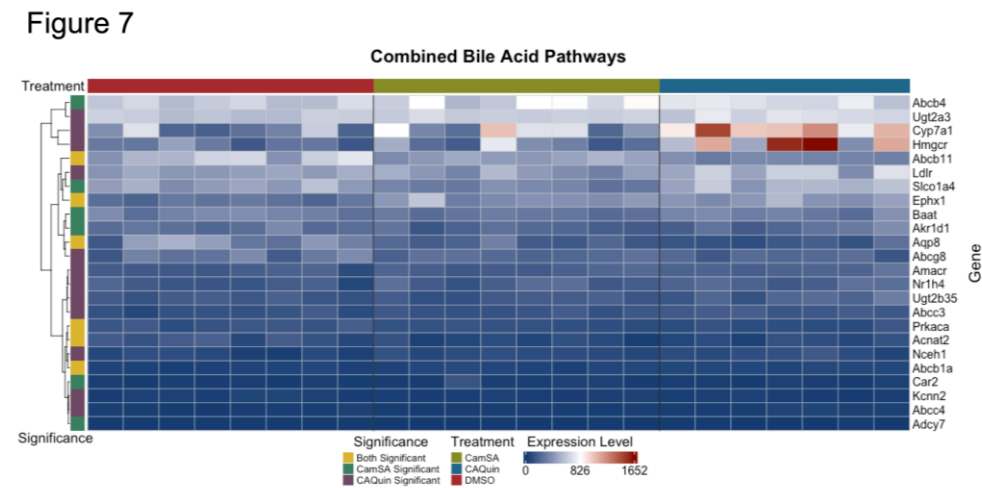


Figure 7. Expression pattern of genes involved in bile synthesis and secretion pathways. Showing here are normalized expression (normalized gene count) levels of significant (unadjusted $p < 0.05$) DEGs between at least one treatment group and the control group. Statistical significance (unadjusted $p < 0.05$) in each treatment was indicated by the color bar on the left.

In CA-Quin-treated mice *Cyp7a1*, *Cyp8b1*, *Amacr*, and *Fxr* (*Nr1h4*) were upregulated while *sCyp2c70* and *Bsep* (*Abcc11*) were downregulated. Similarly, in CamSA-treated mice *Cyp2c70*, *Baat* and *Bsep* were downregulated. All genes from the KEGG pathways are shown in supplementary Figure S5. The normalized counts for the genes in the respective treatment group are shown in supplementary Figure S6.

3.4. qPCR quantification of BA metabolism and transporter genes in the liver and ileum

To validate the transcriptome results, qPCR was performed for selected genes involved in bile synthesis and transport. *Cyp7a1* expression was significantly increased in CA-Quin-treated animals, while *Bsep* (*Abcb11*) was significantly decreased in both treatment groups. Similar to the transcriptomic data, qPCR showed that expression of *Cyp27a1*, *Cyp7b1*, and *Fxr* (*Nr1h4*) was not altered by either CamSA or CA-Quin (Figure 8A).

EHC transport of bile involves numerous proteins in the ileum. Our qPCR data shows that *Ostβ*, a key transporter involved in bile resorption [53], was significantly downregulated in the ileum from the CamSA-treated mice (Figure 8B).

Figure 8

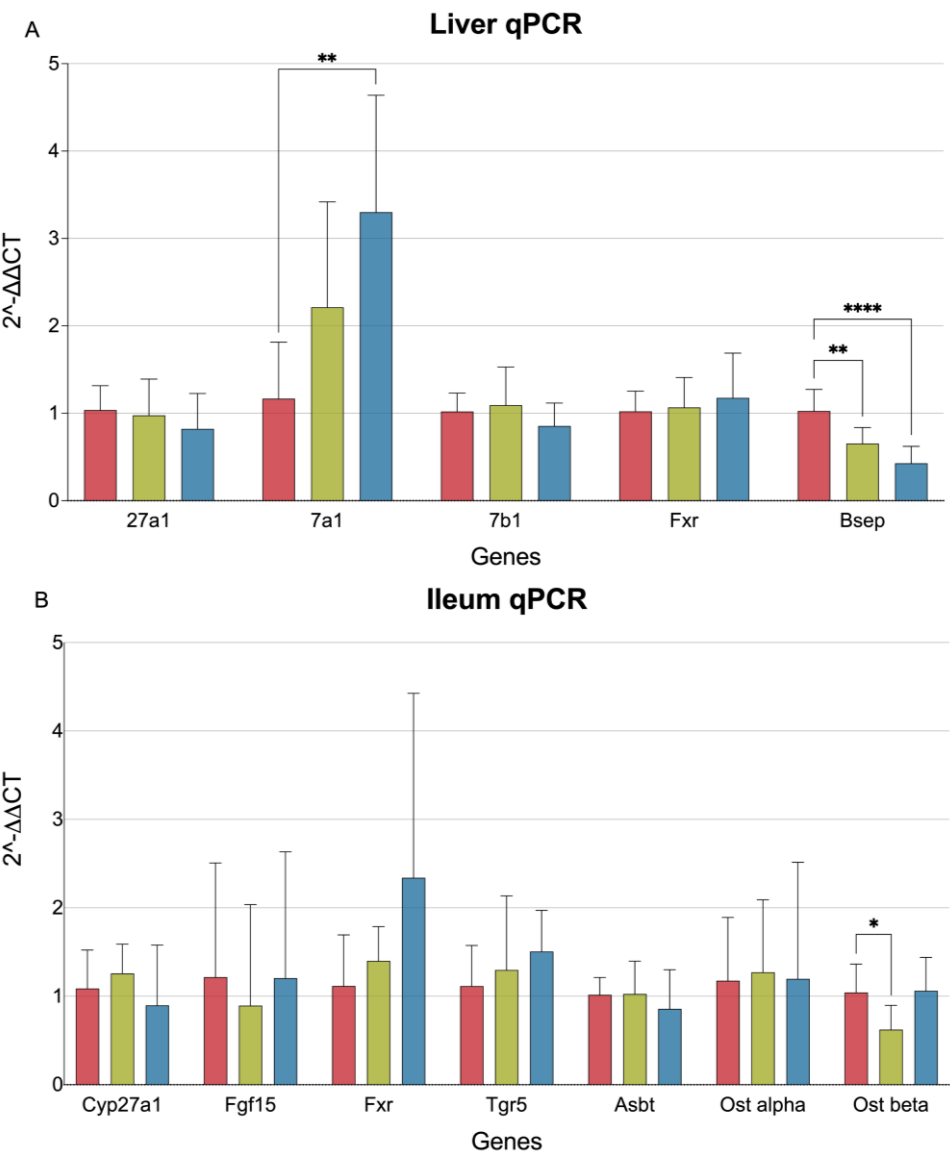


Figure 8. Relative expression level quantified with qPCR of genes involved in bile synthesis, transport, and signaling. Statistics was based on the post hoc Tukey pair-wise test under One-way ANOVA. (**** <0.0001, *** <0.001, ** <0.01, * <0.05).

4. Discussion.

Previously, we have shown that our first generation germination inhibitor, CamSA, can be maintained at therapeutic levels due to recycling via the EHC[14, 54, 55]. However, CamSA can be hydrolyzed by the gut bacteria and becomes inactive and can potentially release toxic byproducts [54]. In addition, one of our other parent inhibitors, CA-Quin, has shown significant potency without being metabolized by the murine microbiota (data not shown). While both compounds do not show obvious signs of toxicity, detailed studies on hepatotoxicity and the effects on bile acids have not been performed. To address this issue, we conducted a transcriptomic and bile acid acids metabolomic analysis on mice treated with the vehicle (DMSO) and the inhibitors, CamSA and CA-Quin for 7 days.

The minimal alteration of hepatic transcriptome by both treatments as demonstrated in the PCA plots indicates that the potential hepatic toxicity of these two compounds is very limited. We also found that the DEG patterns associated with both treatments are largely shared, indicating that the two compounds may have a highly similar pharmacokinetic and pharmacodynamic profile. Most significant changes based on fold change and adjusted p values were only observed in a few genes. Among those, *Cyp3a11* expression is noted to be related to changes in the gut microbiome, cholesterol and bile synthesis, high fat diet, and drug metabolism [56-59] [60]. The *Sgk2* gene is a serine/threonine kinases, that was demonstrated to be related to PI3K-AKT signaling and stimulator for ion channels [61-67]. This signaling pathway can play a role in cholesterol metabolism as well [68, 69]. The *Tubb2a* and *Tuba4a* genes are involved numerous pathways from various transport pathways to immune response related pathways [70-78]. *Ces2a* is known to be part of xenobiotic metabolism and fatty acids/lipids metabolism pathways [79-86]. *Nrep* was shown to be involved in wound healing, TGF- β signaling, and lipid related pathways [87-94]. Altogether, the significant up/down regulation of the most significantly affected genes is not noted to be specific to hepatotoxicity.

While both compounds are BA analogs, our previous study has demonstrated that CamSA enters the EHC. It is thus logically hypothesized that both compounds may interfere with the EHC and homeostasis of BAs. Examination of samples from mice treated with these bile salt analogs demonstrated a few interesting patterns. First, the overall alteration of all BAs among different tissues are highly similar between the CamSA and CA-Quin treatment compared to the DMSO control, which further suggests a similar impact of the two compounds on BA homeostasis.

We also found that the major changes in BA profiles associated with both treatments are the increased BA levels, largely attributed to unconjugated primary and secondary species, among fecal samples, with a high similarity between the samples collected at the two timepoints, suggesting an increased excretion of BAs following both treatments. This trend may suggest a decreased re-absorption of BAs from the intestine back to the liver. This is further supported by the reduced levels of a few secondary BA species in the liver and decreased transcription of the transporter *Ost β* in the CamSA-treated ileum samples.

Reduced BA re-absorption by the EHC may signal to the liver to increase production of BAs. Because BAs are sterol-derivatives, the net effect would be an elevated need of cholesterol usage. This is evidenced by the significant upregulation of hepatic expression of *Cyp7a1* (the rate-limiting enzyme controlling the conversion of cholesterol to BA) and *Hmgcr* (rate limiting enzyme for cholesterol biosynthesis). Furthermore, the downregulation of *Bsep* perhaps also reflects the tendency of BA retention in the liver. Therefore, both CamSA and CA-Quin likely inhibit BA re-absorption and promote intestinal BA excretion.

More detailed analysis of the BA profiles further demonstrated that intestinal TCA and CDCA levels are significantly altered by the two treatments. TCA and CDCA were demonstrated to contribute to the activation and inhibition of *C. difficile* germination, respectively [19, 95]. In this study, we found that the TCA level was significantly reduced in both the chyme and feces samples of the CamSA-treated mice, while CDCA level was significantly increased in the chyme of CA-Quin group, and in feces samples of both treatment groups. Therefore, our data accounts for, at least in part, the inhibitory effects of the two compounds in treating CDI.

In a previous pharmacokinetic analysis of CamSA, we found that it is hydrolyzed into CA and metanilic acid (mSA) [54]. Thus, the increase in CA level observed in the CamSA treated mice, may be due to the hydrolysis of the drug. However, we have shown that CA-Quin is not hydrolyzed by the gut bacteria and remains unchanged in the gut (data not shown). Even though there is an increased level of CA in the ileum of CA-Quin-treated mice as well as in feces samples of both treatments, this increased CA is more likely attributed to the reduced reabsorption. Nevertheless, increased CA in the gut may be also beneficial in treating CDI, as it has been found that CA can bind to one of the *C. difficile* toxins, TcdB, and reduce its toxic effects [96].

Despite no direct observation, our data also indicates an altered gut microbiome following the two treatments. We previously reported that 10 days of treatment with CamSA in uninfected animals

resulted in significantly different microbiome diversity [55]. The BA profile changes shown here are consistent with those prior microbiota observations. Because both de-conjugation of primary BAs and the production of secondary BAs rely on bacterial enzymes [8, 96-98], the increased level of de-conjugated primary and unconjugated secondary BAs in the feces of treated groups likely reflect the increased beta diversity of the gut microbiota as compared to the control group. Indeed, the metabolization of primary BAs into secondary BAs can only be done in certain bacterial species that have the necessary enzymes, such as 7-alpha-dehydroxylases in bacteria like *C. scindens*, *P. hiranonis*, or *E. hylemonae* [99] [97, 100]. Therefore, the significantly increased secondary BAs in the feces may reflect the enrichment of these species which may directly compete with *C. difficile*. Previous studies have shown that *C. scindens* is associated with the prognosis of *C. difficile* infection [96, 97]. Therefore, the retention of these products in the gut may also contribute to the therapeutic effect of the two compounds. Furthermore, secondary bile acids produced in the gut such as DCA or LCA can act as natural inhibitors for *C. difficile* infection [100]. In addition to these changes in BA profiles, the hepatic transcriptomic data demonstrated shared enrichment in GO pathways of monocarboxylic acids and organic acids. It is known that short-chain fatty acids (SCFAs) are primarily produced by gut microbiota. This hepatic transcriptome signature may imply an increased production of SCFAs following the two treatments. Additionally, the hepatic transcriptome data demonstrated shared enrichment in the KEGG pathway, peroxisome. It is known that peroxisomes are important for the beta-oxidation of bile acid precursors[101, 102]. Taken together, our data suggests that both compounds demonstrated anti-*C. difficile* activity via altering the BA-gut microbiome homeostasis and interaction.

5. Conclusions

In summary, our data from our previous studies in potency and pharmacokinetics, in conjunction with the results from the present study, indicate that bile acid analogs are promising therapeutics for *C. difficile* infection. Our study provided further evidence that these analogs are safe, and their unique inferences with the bile acids EHC may normalize the gut microbiota and modulate the profiles of key bile acids that critically impact the *C. difficile* activity.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: All Bile Species Among All Samples (Percent Composition), Figure S2: Enrichment analysis of CamSA DEGs, Figure S3: Enrichment analysis of CA-Quin DEGs, Figure S4: Enrichment analysis of Shared DEGs, Figure S5: Heatmap of Bile Related Genes, Figure S6: RNA Seq Normalized Counts for Significant Bile Related Genes; Table S1: Materials/Reagents, Table S2: Primer Sequences, Table S3: Categorization of Bile Species, Table S4: Quantities of Bile Acids Present;

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Abbreviations

The following abbreviations are used in this manuscript:

12-keto-LCA	2-keto-lithocholic acid
3-keto,7 α ,12 α (OH) ₂	3-keto-7 α ,12 α -dihydroxy-5 β -cholan-24-oic acid
3-keto-LCA	3-keto-lithocholic acid
7-keto-DCA	7-keto-deoxycholic acid
7-keto-LCA	7-keto-lithocholic acid
C4	7 α -hydroxy-4-cholesten-3-one
allo-isoLCA	allo-isolithocholic acid
α -MCA	alpha-muricholic acid
β -MCA	beta-muricholic acid
BA	bile acid
CDCA	chenodeoxycholic acid
CDCA-3-S	chenodeoxycholic acid-3-sulfate
CA	cholic acid
CA-3-S	cholic acid-3-sulfate
CA-7-S	cholic acid-7-sulfate
CDI	<i>Clostridioides difficile</i> infection
DCA	deoxycholic acid
DCA-3-S	deoxycholic acid-3-sulfate
DMSO	dimethyl sulfoxide
EHC	enterohepatic circulation
G β -MCA	glyco-beta-muricholic acid
GCDCA	glycochenodeoxycholic acid
GCA	glycocholic acid
GDCA	glycodeoxycholic acid
GHCA	glycohyocholic acid
GHDCA	glycohyodeoxycholic acid
GLCA	glycolithocholic acid
GUDCA	glycoursodeoxycholic acid
HCA	hyocholic acid
HDCA	hyodeoxycholic acid
isoDCA	isodeoxycholic acid
isoLCA	isolithocholic acid
LC-MS	liquid chromatography- mass spectrometry
LCA	lithocholic acid
LCA-3-S	lithocholic acid-3-sulfate

mSA	metanilic acid
MDCA	murideoxycholic acid
ω -MCA	omega-muricholic acid
PCA	principle component analysis
T α -MCA	tauro-alpha-muricholic acid
T β -MCA	tauro-beta-muricholic acid
T ω -MCA	tauro-omega-muricholic acid
TCDCA	taurochenodeoxycholic acid
TCA	taurocholic acid
TCA	taurodeoxycholic acid
THDCA	taurohyodeoxycholic acid
TLCA	tauroolithocholic acid
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UDCA-3-S	ursodeoxycholic acid-3-sulfate

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