Ursodeoxycholic acid and its taurine/glycine conjugated species reduce colitgenic dysbiosis and equally suppress experimental colitis in mice.

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Running title: UDCA and its conjugates reduce colitogenic dysbiosis.

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ABSTRACT

The promising results with secondary bile acids in experimental colitis suggest that they may represent an attractive and safe class of drugs for the treatment of inflammatory bowel diseases (IBD). However, the exact mechanism by which bile acid therapy confers protection from colitogenesis is currently unknown. Since the gut microbiota plays a crucial role in the pathogenesis of IBD, and exogenous bile acid administration may affect the community structure of the microbiota, we examined the impact of the secondary bile acid ursodeoxycholic acid (UDCA) and its taurine/glycine conjugates on the fecal microbial community structure during experimental colitis. Daily oral administration of UDCA, tauroursodeoxycholic acid (TUDCA) or glycoursodeoxycholic acid (GUDCA) equally lowered the severity of dextran sodium sulfate-induced colitis in mice, as evidenced by reduced body weight loss, colonic shortening and expression of inflammatory cytokines. Illumina sequencing demonstrated that bile acid therapy during colitis did not restore fecal bacterial richness and diversity. However, bile acid therapy normalized the colitis-associated increased ratio of Firmicutes to Bacteroidetes. Interestingly, administration of bile acids prevented the loss of Clostridium cluster XIVa and increased the abundance of Akkermansia muciniphila, bacterial species known to be particularly decreased in IBD patients. We conclude that UDCA, which is an FDA-approved drug for cholestatic liver disorders, could be an attractive treatment option to reduce dysbiosis and improve inflammation in human IBD.

IMPORTANCE

Secondary bile acids are emerging as attractive candidates for the treatment of inflammatory bowel disease. Although bile acids may affect the intestinal microbial community structure, which significantly contributes to the course of these inflammatory disorders, the impact of bile
acid therapy on the fecal microbiota during colitis has not yet been considered. Here, we studied the alterations in the fecal microbial abundance in colitic mice following the administration of secondary bile acids. Our results show that secondary bile acids reduce the severity of colitis and improve colitis-associated fecal dysbiosis at the phylum level. This study indicates that secondary bile acids might act as a safe and effective drug for inflammatory bowel disease.

**INTRODUCTION**

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract characterized by intestinal dysbiosis. Restricted bacterial diversity and underrepresentation of anti-inflammatory microorganisms such as *Clostridium* cluster XIVa and *Akkermansia muciniphila* represent typical dysbiotic features in IBD (1–4). Since the intestinal microbial community performs a wide range of bile acid modifications including deconjugation, dehydroxylation, oxidation and epimerization (5), shifts in the composition of the gut microbiota are associated with perturbations of the fecal bile acid profile (6, 7). Of particular interest, Duboc and colleagues demonstrated that the conversion of primary bile acids (synthesized in the liver from cholesterol) to secondary bile acids (generated by bacterial modifications) is impaired in IBD patients (7). Because secondary bile acids exhibit immunomodulatory functions (7–10), increasing secondary bile acid levels in the intestinal lumen could be an efficient therapeutic approach for IBD. In line with this hypothesis, the administration of the secondary hydrophilic bile acid ursodeoxycholic acid (UDCA) ameliorates experimental colitis but the exact mechanism protecting from colitogenesis is not fully understood (11).

When administered orally, unconjugated UDCA is rapidly conjugated with glycine in humans, and to a lesser extent with taurine, on its first pass through the liver (12, 13). Based on the observation that fecal bile acid hydrophobicity correlates with the severity of colitis (14), it is
reasonable to assume that conjugates of UDCA, which are more hydrophilic than unconjugated UDCA, might be more favorable therapeutic agents for intestinal inflammation. In this regard, we and others have shown that tauroursodeoxycholic acid (TUDCA) alleviates dextran sodium sulfate (DSS)-induced colitis in mice (15, 16). The potential beneficial effect of glycoursoodeoxycholic acid (GUDCA) in colitis, however, has not been addressed so far and studies comparing the therapeutic effectiveness of these different bile acid species are lacking.

While the composition of the luminal bile acid pool is controlled by intestinal bacteria, it is well established that bile acids, in turn, also shape the gut microbiota. Bile acids restrict bacterial proliferation and overgrowth directly by causing membrane damage, which is positively correlated with bile acid hydrophobicity (17–19). Thus, the bactericidal activity of bile acids decreases with increasing numbers of hydroxyl groups and by conjugation of the bile acid side chain with taurine or glycine (19). In addition to their role as antimicrobial agents, bile acids also stimulate the growth of selected bacterial species (5). Similarly, these properties are determined both by the hydroxylation pattern and the conjugation status of the bile acid steroid nucleus. For example, increased intestinal levels of bile acids carrying a hydroxyl group at position C7 of the steroid core favor the growth of 7α-dehydroxylating bacteria, such as Clostridium cluster XIVa members (20, 21). Furthermore, the amino acids in conjugated bile acids act as microbial substrates for distinct bacterial groups; glycine is metabolized by Clostridium species (22, 23), while taurine is a source of sulphite from which Bilophila wadsworthia derives energy for its growth (24, 25). Interestingly, a diet high in saturated fat promotes taurine-conjugation of hepatic bile acids, resulting in the outgrowth of B. wadsworthia and exacerbation of colitis (26).

Considering that the gut microbial architecture and metabolism contribute to the course of IBD (27, 28), we compared the therapeutic effectiveness of UDCA and its taurine/glycine conjugates in DSS-induced colitis in mice and investigated their impact on the fecal microbial community.
MATERIALS AND METHODS

Animals. Male 8-week-old C57Bl/6J mice were obtained from Harlan (Harlan Laboratories, Horst, The Netherlands) and maintained under standard laboratory conditions with *ad libitum* access to food (mice maintenance chow, Carfil Labofood, Pavan Service, Belgium) and water. Prior to the experiment, mice were co-housed to homogenize gut microbiota between experimental groups. After a one-week acclimatization, mice were assigned to the treatment groups based on body weights. In order to avoid bacterial cross-contamination between groups, mice of different treatment groups were housed in separate cages. The study was approved by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University (ECD 2014-25).

Bile acid treatment. Mice were divided into five groups (n = 8 in each group). Three of them received bile acid treatment: UDCA (Tokyo Chemical Industry Co. Ltd, Toshima-Ku, Tokyo, Japan), TUDCA (Calbiochem, Darmstadt, Germany) or GUDCA (Sigma-Aldrich, Diegem, Belgium). Bile acids were dissolved in phosphate-buffered saline (PBS) or Labrafil® M1944 (Gattefosse, Saint-Priest Cedex, France) and administered daily by oral gavage (500 mg/kg/day). Treatments started at day 0 of DSS exposure. A non-DSS control group and DSS control group (referred to as placebo-treated group) received the vehicle (PBS or Labrafil®) alone.

Induction and assessment of colitis. Acute colitis was established by adding 4% (w/v) DSS (molecular weight 36,000–50,000; MP Biomedicals, Illkirch, France) to the drinking water for 7 days, followed by normal water for 3 days. A non-DSS control group received normal drinking water throughout the experiment. Body weight and disease activity were recorded daily. A disease activity index (DAI) was calculated as the combined score of body weight loss (0, none; 1, 0-10%; 2, 10-20%; 3, >20%), stool consistency (0, normal droppings; 1, loose droppings; 2, ...
diarrhea) and fecal blood loss (0, none; 1, hemoccult positive; 2, gross bleeding). Occult blood
was detected using the Coloscreen Hemoccult kit (Helena Laboratories Inc., Beaumont, Texas,
USA). Ten days after initiation of the experiment, mice were anesthetized and blood was
collected from the retro-orbital sinus. The mice were then sacrificed by cervical dislocation, the
colons were removed and their lengths were measured. Segments of distal colon were cut, rinsed
with PBS and frozen in liquid nitrogen. The blood was centrifuged (10,000 rpm for 10 min at
4°C) and serum was collected. All samples were stored at -80°C until further processing.

**Luminex.** Colonic tissues were homogenized in PBS containing protease and phosphatase
inhibitors and total protein concentration was measured using the Bradford method (Bio-Rad,
Nazareth, Belgium). Protein levels of chemokine (C-X-C motif) ligand 1 (CXCL1), granulocyte
colony-stimulating factor (G-CSF) and interleukin (IL)-6 were determined in colon homogenates
and serum using the Bio-Plex Pro Mouse Cytokine Group I multiplex kit (Bio-Rad), according to
the manufacturer’s instructions. Measurements were performed with the Bio-Plex MAGPIX
Multiplex Reader and data were analyzed using the Bio-Plex Manager 6.1 software (Bio-Rad).

**DNA extraction from fecal samples.** Fresh fecal pellets were collected at day 9 of colitis and
immediately stored at -80°C. Total DNA was extracted from the fecal samples using the QIAamp
DNA Stool Mini Kit (Qiagen Benelux, Venlo, The Netherlands). First, 180 to 220 mg of stool
was resuspended in 1.4 ml buffer ASL. Then, 0.5 g 0.1 mm Zirconia beads (Biospec Products,
Bartlesville, Oklahoma) and 4 glass beads (Biospec Products) were added and samples were
homogenized by vortexing. The suspension was then heated at 95°C for 15 min and the
manufacturer’s instructions were followed.

**Illumina sequencing.** The V1-2 region of the 16S rRNA gene was amplified as previously
described (29). Briefly, in a first 20 cycle polymerase chain reaction (PCR) reaction, the 16S
rRNA gene target was enriched using the well-documented 27F and 338R primers (30, 31) as
previously specified (32). This reaction mixture was used as template in a second 15 cycle PCR reaction with primers comprising sequences complementary to the Illumina specific adaptors to the 5'-ends (29). The latter reaction mixture was then used as template in a third 10 cycle PCR reaction with primers designed to integrate both the sequence of the specific Illumina multiplexing sequencing primers and the index primers. Libraries prepared by pooling equimolar ratios of amplicons were finally sequenced on a MiSeq (Illumina, Hayward, CA, USA). Afterwards, reads were annotated as described by Verstraelen et al. (33).

**Illumina data analysis.** Data-analysis was performed as previously described (33). After resampling to the minimum sequencing depth using the phyloseq package (34) from the R program (35), a total of 8,911 reads were obtained. Rarefaction curves were generated using the vegan package from R (36). All phylotypes were assigned a taxonomic affiliation based on the naive Bayesian classification (RDP classifier) (37) with a threshold of 80%. Relative abundances of all phylotypes were then compared between different experimental groups.

**Quantitative real-time PCR (qRT-PCR).** Total fecal DNA was diluted 1:2 in water and 3 µl was used in qRT-PCR with SYBR Green (SensiMix™ SYBR No-ROX Kit, Bioline Reagents, UK) and 250 nM of each primer (BioLegio, Nijmegen, The Netherlands). Primer sequences used for amplification of *A. muciniphila* were 5'-CAGCACGTGAAGGGGGAC-3' and 5'-CCTTGCGGTTGGCTTCAGAT-3' (38). A two-step program was performed on the LightCycler 480 (Roche). Cycling conditions were 95°C for 10 min, 45 cycles of 95°C for 10 s and 60°C for 1 min. The amount of *A. muciniphila* 16S rRNA gene in each sample was normalized to the total amount of bacterial 16S rRNA gene. For the quantification of total 16S rRNA gene copies, fecal DNA was diluted 1:10 in water and the universal bacterial 16S rRNA gene primers PRBA338f 5’-ACTCCTACGGGAGGCAGCAG-3’ and PRUN518r 5’-ATTACCAGGCTGCTGG-3’ were
Bile acid quantification.

Sample preparation. Fresh fecal pellets were collected at day 4 of colitis and immediately stored at -80°C. Before bile acids were extracted from fecal samples, 20 µl of an internal standard solution (TUDCA-d5 (Santa-Cruz Biotechnology, Heidelberg, Germany) at 25 ng/µl in methanol (VWR International, Merck Millipore, Darmstadt, Germany)) was added to 25 mg feces. The extraction protocol started with the addition of 5 ml ice-cold acetonitrile (VWR International) containing 5% ammonium hydroxide (Merck Millipore, Darmstadt, Germany). The solution was homogenized with an Ultra-Turrax homogenizer, thoroughly mixed by vortexing for 1 min and then placed in an ultrasonic bath for 30 min. The resultant mixture was centrifuged at 9,000 x g for 10 min and supernatant was collected. The extraction procedure was repeated once more and the combined supernatants were subsequently evaporated under nitrogen at 40°C. Each dry extract was then resuspended in 200 µl of a 40:60 mixture of solvent A (7.5 mM ammonium acetate (Merck Millipore) in ultrapure water, pH 4.0) and solvent B (5% acetonitrile in methanol), centrifuged at 9,000 x g for 10 min and supernatant was collected. To compensate for matrix effects, the standard addition method was applied for bile acid quantification (40). Briefly, supernatant was divided into two equal aliquots and transferred to liquid chromatography-mass spectrometry vials. One aliquot was spiked with 20 µl of the 40:60 mixture of solvent A and B. The other aliquot was spiked with 20 µl of bile acid solution (a 40:60 mixture of solvent A and B, supplemented with 16.5 ng/µl lithocholic acid (LCA; Sigma-Aldrich), 89.4 ng/µl UDCA (Sigma-Aldrich), 0.75 ng/µl TUDCA (Calbiochem) and 0.75 ng/µl GUDCA (Sigma-Aldrich)). A 10 µl aliquot of each sample was injected into the ultra-high performance liquid chromatography with high resolution mass spectrometry (UHPLC-HRMS) system.
**UHPLC-HRMS analysis.** Chromatographic separation of bile acids was carried out on an Accela UHPLC system of Thermo Fisher Scientific (San José, CA, USA), with an Acquity UPLC HSS C18 column (1.8 μm, 50 mm × 2.1 mm, Waters). The binary solvent system consisting of two solvents A and B was set at a constant flow rate of 300 µl/min at 35°C. For elution, a gradient profile was applied with the following proportions (v/v) of solvent A: 0 – 1.0 min at 40%, 1.0 – 6.0 min from 40% to 1%, 6.0 – 8.0 min at 1%, 8.0 – 8.1 min from 1% to 40%, followed by 3.9 min of re-equilibration.

HRMS analysis was performed on an Exactive™ stand-alone benchtop mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionization source (HESI-II), operating in the negative ionization mode. Ionization source working parameters were optimized and were set to a sheath, auxiliary and sweep gas of 40, 5 and 1 arbitrary units (au), respectively, heater and capillary temperature of 120°C and 375°C and tube lens, skimmer, capillary and spray voltage of 123 V, 22 V, 43.5 V and 4 kV (+/-), respectively. A scan range of \( m/z \) 300-550 was selected and the resolution was set at 100,000 FWHM at 1 Hz (1 scan per second). The automatic gain control (AGC) target was set at high dynamic range (3 × E⁶ ions) and the maximum injection time was 100 ms.

**Data processing.** HRMS data processing was performed with Xcalibur™ 3.0 (Thermo Fisher Scientific). The concentration of a selected bile acid was calculated using the following formula

\[
C_{\text{unk}} = \frac{C_{\text{SA}} \times A_{\text{Runk}}}{A_{\text{RSA}} - A_{\text{Runk}}}
\]

with \( C_{\text{unk}} \) being the unknown concentration of the bile acid in the original fecal sample, \( C_{\text{SA}} \) being the spiked concentration of the bile acid in the fecal sample after standard addition, \( A_{\text{Runk}} \) being
the area ratio of the bile acid in the original sample and AR_{SA} being the area ratio of the bile acid
in the fecal sample after standard addition.

Statistical analysis. Statistical analysis was performed using SPSS Statistics version 22.0 (IBM
SPSS, Chicago, USA) and GraphPad Prism version 4 (GraphPad, California, USA). All data are
expressed as mean ± SEM. Data were tested for normality using the Kolmogorov-Smirnov test.
Statistical significant differences between groups were assessed using the unpaired Student’s t-
test for normally distributed data, applying the Welch’s correction in case of unequal variances,
or the Mann-Whitney U test for non-normally distributed data. Two-tailed probabilities were
calculated and P-values of less than 0.05 were considered statistically significant.

Nucleotide sequence accession number. The sequence data were submitted to the European
Nucleotide Archive (ENA) with accession numbers from LT700235 to LT702885 and can be
accessed at http://www.ebi.ac.uk/ena/data/view/LT700235-LT702885.

RESULTS

Oral administration of UDCA or its taurine/glycine conjugated species is equally protective
in acute DSS-induced colitis

To compare the therapeutic effects of UDCA and its taurine/glycine derivatives on DSS-induced
colitis, C57BL6/J mice were challenged with 4% DSS for 7 days and treated daily with UDCA,
TUDCA or GUDCA by oral gavage. Bile acid therapy reduced the rate of body weight loss, with
no differences in efficacy between the three bile acid treatments (Figure 1A). At day 10 after the
initiation of colitis, body weight loss was significantly higher in placebo-treated mice than in bile
acid-treated mice (P=0.027; P=0.021 and P=0.021 for treatment with UDCA, TUDCA and
GUDCA, respectively). Accordingly, the clinical disease activity score, colonic shortening and
colonic concentrations of CXCL1, G-CSF and IL-6, which have been reported to show enhanced
expression in the acute phase of DSS-induced colitis (41, 42), were all attenuated following bile acid treatment (Figure 1B-D). At the systemic level, lower levels of CXCL1 and G-CSF, but not IL-6, were detected in the serum of bile acid-treated mice as compared with the placebo-treated group (Figure 1E). Together, these data demonstrate that UDCA and its taurine/glycine conjugates decrease the severity of DSS-induced colitis with similar effectiveness.

Bile acid supplementation to mice challenged with DSS prevents colitis-associated dysbiosis at the phylum level

Because bile acids have been recognized as modulators of the intestinal microbiota (21, 26), which is believed to play a critical role in colitis (1, 28), we questioned whether administration of UDCA or its conjugated derivatives prevented dysbiosis during experimental colitis. Fecal samples were collected two days after removing DSS from the drinking water (day 9), when colitis was fully established, and microbiota profiles were determined by 16S rRNA Illumina MiSeq sequencing. Four out of 40 mice were excluded from microbiota analysis because of insufficient amounts of feces available for DNA extraction. As expected, administration of DSS resulted in a significant reduction of species richness (number of operational taxonomic units; P=0.002; Figure 2A) and microbial diversity (Shannon index; takes into account both species abundance and evenness; P=0.002; Figure 2B). In addition, the total fecal bacterial load had dropped significantly to 22% of values seen in non-DSS control mice (P=0.002; Figure 2C). Compared to the placebo-treated group, microbial richness and diversity were slightly declined when colitic mice were treated with UDCA (P=0.086 and P=0.015, respectively), but no changes could be observed after the administration of TUDCA or GUDCA. Furthermore, daily administration of UDCA or its taurine/glycine conjugates during colitis did not affect the decrease in fecal bacterial load (Figure 2A-C).
We next analyzed the fecal microbial composition at the major taxonomic hierarchy levels to determine if particular bacterial phyla were altered following bile acid therapy during active disease. Taxonomic assignment of the sequence reads revealed two dominant phyla, *Bacteroidetes* and *Firmicutes*, accounting for respectively 58.31% and 31.05% in fecal communities of non-DSS control mice. Other phyla present with an average relative abundance ranging from 4.50% to 0.09% were *Proteobacteria*, *Deferribacteria*, *Candidatus Saccharibacteria* and *Actinobacteria*. Administration of DSS significantly altered the structure of the fecal microbiome. At day 9 of colitis, the *Firmicutes/Bacteroidetes* ratio increased in mice that were challenged with DSS (Figure 2D). More specifically, bacteria of the *Bacteroidetes* phylum were underrepresented in fecal samples of placebo-treated mice while the relative abundance of *Firmicutes* bacteria tended to increase (Figure 2E). Interestingly, oral administration of TUDCA or UDCA normalized the *Firmicutes/Bacteroidetes* ratio, while GUDCA treatment showed a slight but non-significant tendency to reduce this ratio (P=0.12, Figure 2D). Compared to placebo-treated mice, the fecal microbial community of bile acid-treated mice with colitis showed a lower abundance of *Firmicutes* and an increased abundance of *Bacteroidetes* (Figure 2E). These results show that bile acid therapy in DSS-induced colitis neither mitigate nor aggravate altered microbial richness and population diversity but corrects fecal microbiota dysbiosis at the phylum level.

**Bile acid supplementation to mice challenged with DSS alters the fecal microbiota at lower taxonomic levels**

To examine which bacterial populations accounted for the changes at phylum level, we investigated the relative abundance of bacteria at lower taxonomic levels. The abundance of unclassified *Bacteroidetes* was reduced upon DSS administration, whereas no changes were
noted for *Bacteroidia* levels (Figure 3A). Interestingly, bile acid supplementation did not elicit an
effect on unclassified *Bacteroidetes* but was associated with a significant increase in the
abundance of *Bacteroidia* compared with placebo-treated mice (Figure 3A). Examination at the
family level indicated that upon DSS challenge, the relative proportion of *Bacteroidaceae* and
*Porphyromonadaceae* increased (Figure 3B and C), while *Prevotellaceae* family members nearly
completely disappeared in fecal samples of placebo-treated mice (0.03% vs. 6.64% in non-DSS
control mice, Figure 3D). Interestingly, the relative abundance of *Prevotellaceae* increased to
2.24% and 2.61% when mice were treated with UDCA or TUDCA, respectively (Figure 3D).
Moreover, *Bacteroidaceae* tended to further increase upon bile acid supplementation, especially
when mice were treated with UDCA (Figure 3B). Within *Firmicutes*, *Erysipelotrichia* expanded
in the fecal samples of mice that were challenged with DSS, though bile acid therapy did not
abrogate nor amplify this increase (Figure 3A). However, the relative abundance of *Clostridia*
remained unchanged in the placebo-treated group, but declined in mice that were administered
UDCA or derivatives (Figure 3A). Comparisons at the genus level further demonstrated that
DSS-induced colitis was associated with the outgrowth of *Clostridium* clusters XI and XIVb and
with a depletion of *Clostridium* cluster XIVa (Figure 3E; data not shown). As compared to the
placebo-treated group, colitic mice that were treated with UDCA or conjugates exhibited
increased numbers of *Clostridium* cluster XIVa bacteria (Figure 3E). *Clostridium* species
belonging to the clusters XI and XIVb, however, were not altered upon bile acid treatment (data
not shown). Interestingly, sequences that were aligned to the *Verrucomicrobia* phylum were
completely absent in the fecal communities of non-DSS control mice. Within this phylum,
however, one genus (*Akkermansia*) expanded in the fecal samples of mice that were challenged
with DSS. *Akkermansia* accounted for 0.047% of the detectable bacteria in the fecal samples of
placebo-treated mice and the relative abundance further increased upon treatment with UDCA,
TUDCA or GUDCA (0.145%, 0.114% and 0.159%, respectively; Figure 3F). Given the importance of these species in IBD (3, 4), the bile acid-induced enrichment of *A. muciniphila* was confirmed by qRT-PCR analysis (Figure 3G).

**Oral administration of UDCA, TUDCA and GUDCA results in a similar fecal bile acid pool**

To compare the extent of biotransformation of orally administered UDCA, TUDCA and GUDCA, we performed UHPLC-HRMS analysis on fecal samples collected at day 4 of colitis. This time point was chosen because changes in the bile acid composition have been suggested to reach a steady-state already within 4 days of bile acid administration (43). Due to insufficient quantities of feces, two mice were excluded from UHPLC-HRMS analysis. UDCA concentrations were significantly higher in fecal samples of mice administered UDCA or its taurine/glycine conjugates than in those of placebo-treated mice (Figure 4A). Concomitant with these changes, we observed increased fecal levels of TUDCA and LCA in bile acid-treated mice (Figure 4B and C). Of note, these changes occurred irrespective of whether animals were treated with UDCA, TUDCA or GUDCA and without significant differences between the three bile acid treatments. In contrast, administration of GUDCA caused a substantial increase in fecal GUDCA levels, while no elevation was observed in animals that were administered UDCA or TUDCA (Figure 4D). This finding is consistent with the fact that bile acids are predominantly conjugated with taurine (>97%), instead of glycine (<0.1%), in mice (44). Together, these data indicate that orally administered UDCA, TUDCA and GUDCA are extensively metabolized *in vivo*, resulting in a similar fecal bile acid composition.

**DISCUSSION**
Previous studies have reported the therapeutic potential of the hydrophilic bile acids UDCA and TUDCA in experimental colitis (11, 15, 16), albeit without comparing their respective effectiveness. In the present study, we showed that daily administration of UDCA and its taurine- and glycine-coupled conjugates equally attenuated body weight loss, disease activity and colonic shortening caused by DSS. Moreover, oral bile acid therapy reduced pro-inflammatory cytokine concentrations in the colon and serum to a similar extent.

Bile acids are important regulators of the intestinal microbiota (21), which plays a crucial role in the pathogenesis of IBD (27). In order to examine the role of UDCA and its taurine/glycine conjugated species in the regulation of the intestinal microbiota during colitis, we orally administered UDCA, TUDCA or GUDCA to mice that were challenged with DSS and determined fecal microbiota profiles by 16S rRNA Illumina MiSeq sequencing. Consistent with previous studies, DSS-induced colitis was associated with distinct alterations in the population structure of the gut microbiota (45, 46). These changes were generally characterized by a reduced overall microbial diversity and a decrease in species richness and bacterial load, which are also main hallmarks of dysbiosis in IBD patients (47, 48). We showed that oral bile acid administration did not prevent the DSS-induced changes in microbial richness, diversity, and bacterial load. Interestingly, species diversity decreased even more when colitic mice were treated with UDCA. However, although reduced richness and diversity of the gut microbiota have been associated with human disease (49–51), it seems unlikely that this effect is also clinically relevant; the clinical outcome in UDCA-treated mice was similar to that of mice that were treated with TUDCA or GUDCA.

At the phylum level, TUDCA, UDCA, and to a lesser extent GUDCA, normalized the Firmicutes/Bacteroidetes ratio that was increased in placebo-treated mice with colitis. This ratio is often used as a proxy for microbial health status (52–57) and, more specifically, to describe the...
degree of dysbiosis in IBD (55–57). Of note, the phylum-level population shifts from *Bacteroidetes* to *Firmicutes* induced by DSS in our study resemble those observed in obese individuals and in animals on a high-fat diet, and have been associated with low-grade intestinal and systemic inflammation in obesity (58, 59). In this context, fecal calprotectin and plasma C-reactive protein levels showed a positive correlation with bacteria belonging to the *Firmicutes*, whereas a negative correlation was found between C-reactive protein levels and specific groups within the *Bacteroidetes* (58). It is therefore likely that bile acid therapy counteracts the development of a “pro-inflammatory” microbiota during colitis. This is speculative since it remains unknown if the changes seen in the *Firmicutes/Bacteroidetes* ratio following bile acid treatment are a cause, rather than a consequence, of the bile acid anti-inflammatory effect. However, bile acid therapy did not prevent the DSS-induced decrease in unclassified members of the phylum *Bacteroidetes* but increased the relative abundance of *Bacteroidia*, which was not affected by DSS. Thus, we can speculate that bile acid therapy directly interferes with an imbalanced microbial environment.

We demonstrated that *Clostridium* cluster XIVa species were significantly underrepresented upon DSS challenge, confirming previous observations in both human and experimental IBD (2, 60–62). However, oral administration of UDCA or its taurine/glycine conjugates was able to provoke an enrichment of these species compared with placebo-treated mice. It has been shown that selected members within the clostridial cluster XIVa possess 7α-dehydroxylation activity (20), which is involved in a multistep biochemical pathway converting UDCA to LCA (63, 64). In our experiment, orally administered TUDCA and GUDCA were rapidly deconjugated to UDCA, so either bile acid treatment created a substrate-rich environment for these species. This may explain the bloom of *Clostridium* cluster XIVa that was observed in colitic mice that were treated with bile acids. *Clostridium* spp. belonging to cluster XIVa are important inducers of regulatory T
In addition, 80% of the butyrate-producing strains isolated from human fecal samples belong to the clostridial cluster XIVa (66). Butyrate is a short-chain fatty acid with distinctive anti-inflammatory properties that has already proven its efficacy in Crohn’s disease (67). However, butyrate-producing bacteria are depleted in the fecal microbiota of IBD patients (61, 62, 68). Thus, our observation that UDCA or its taurine/glycine conjugates increased the abundance of *Clostridium* cluster XIVa during colonic inflammation is of particular interest and may suggest an immunomodulatory role of these bile acids.

Another finding of this study was the overrepresentation of *Bacteroidaceae, Prevotellaceae* and *Akkermansia* in fecal samples of bile acid-treated mice following DSS exposure. These results might be related to the stimulatory effect of bile acids on mucin secretion as a defense mechanism to protect the gastrointestinal epithelium against potential bile acid toxicity (69–71). Bacterial species belonging to the genera *Bacteroides*, *Prevotella* and *Akkermansia* produce one or more enzymes required for mucin degradation (72), which is enhanced during the acute phase of DSS-induced colitis (46). Therefore, it is reasonable to assume that these bacteria can grow better in an environment that is, resulting from exogenous bile acid administration, enriched with mucins. *A. muciniphila* is a commensal bacterium residing in the mucus layer of the intestinal tract and has been shown to be reduced in IBD patients (3, 4). Although conflicting results were obtained in studies assessing the role of *Akkermansia* in colonic inflammation, these species are thought to play a key role in the regulation of gut barrier function and mucosal immune responses toward the commensal microbiota (73, 74).

The molecular structure of a bile acid determines its metabolism, physicochemical properties and biological effects (75). In the present study, we used three bile acids sharing the same steroidal hydroxylation pattern but differing in their amino acid conjugation pattern. Neither bile acid species tested proved to be more or less efficacious than the other in reducing colonic...
inflammation. Likewise, administration of UDCA induced similar changes in the bacterial community compared with its taurine/glycine conjugated species. These observations can be explained by the rapid \textit{in vivo} biotransformation of orally administered bile acids by the liver and by the intestinal microbiota. With the exception of fecal GUDCA concentrations, which were only increased following GUDCA therapy, there were no differences in fecal concentrations of UDCA, TUDCA or LCA between mice that were administered UDCA or its conjugates. This is in contrast with data from previous studies in patients with primary biliary cirrhosis (12) and rats (43) showing that, compared with UDCA, orally administered TUDCA undergoes reduced 7-dehydroxylation to LCA. It is conceivable that interspecies differences in intestinal microbiota account for these discrepancies. For example, deconjugation of TUDCA or GUDCA is a prerequisite for further 7-dehydroxylation and is catalyzed by bile salt hydrolases (43). Because \textit{Lactobacilli}, which express bile salt hydrolases, are more abundant in the mouse gut microbiota as compared to the human gut microbiota (76), it is likely that biotransformation of these conjugated bile acids occurs to a larger extent in mice.

In summary, we report that UDCA and its taurine/glycine conjugated species ameliorate colonic inflammation in mice without differing in therapeutic effectiveness, and reduce DSS-induced fecal dysbiosis at the phylum level, irrespective of the bile acid conjugation status. As we demonstrated no advantage of using either the taurine or glycine conjugate of UDCA, we suggest that UDCA could be a safe and readily available treatment option for IBD. This conclusion is further supported by the current therapeutic use of UDCA in cholestatic patients (77) and by its preventive effects on IBD-associated colorectal carcinogenesis (78–80).

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REFERENCES


FIGURE LEGENDS

Figure 1. Oral administration of UDCA, TUDCA and GUDCA improves clinical parameters and inflammatory markers in acute DSS-induced colitis. C57BL/6J mice received 4% DSS in the drinking water for 7 days, followed by normal water for 3 days. Control mice received water alone. From the start of DSS administration, mice were treated with UDCA, TUDCA or GUDCA (500 mg/kg/d) by oral gavage. (A) Body weight changes during acute DSS-induced colitis. Body weights are represented as a percentage of their initial body weight at day 0. Results of a Mann-Whitney U test on day 10 are shown. (B) Clinical disease activity score. Results of a Mann-Whitney U test on day 9 are shown. (C) Colon lengths were assessed upon...
euthanasia on day 10. (D,E) Cytokine levels of CXCL1, G-CSF and IL-6 in colonic tissue (D) and serum (E) collected on day 10. Data are represented as the mean ± SEM (n = 8 in each group). *P<0.05, **P<0.01. C = Control; P = Placebo; T = TUDCA; U = UDCA; G = GUDCA.

Figure 2. Oral administration of UDCA, TUDCA and GUDCA during DSS-induced colitis prevents colitis-associated dysbiosis at the phylum level. Fecal samples were collected on day 9 of colitis and microbiota profiles were characterized by 16S rRNA Illumina MiSeq sequencing. (A-C) Estimation of (A) species richness (i.e., total number of operational taxonomic units), (B) species diversity (i.e., Shannon index) and (C) bacterial load in the fecal microbiota. Bacterial load was calculated as $2^{\Delta \text{CT}}$ / [total DNA concentration]. (D) Ratio of the percentage of 16S rRNA gene sequences belonging to Firmicutes and Bacteroidetes. (E) Composition of the fecal microbial community at the phylum level. Data are represented as the mean ± SEM (n ≥ 6 in each group). *P<0.05, **P<0.01. C = Control; P = Placebo; T = TUDCA; U = UDCA; G = GUDCA.

Figure 3. Oral administration of UDCA, TUDCA and GUDCA during DSS-induced colitis alters the fecal microbiota at lower taxonomic levels. Fecal samples were collected on day 9 of colitis and microbiota profiles were characterized by 16S rRNA Illumina MiSeq sequencing. (A) Composition of the fecal microbial community at the class level. (B-F) Percentage of 16S rRNA gene sequences belonging to (B) Bacteroidaceae, (C) Porphyromonadaceae, (D) Prevotellaceae, (E) Clostridium cluster XIVa and (F) Akkermansia. (G) qRT-PCR results for A. muciniphila. Copy numbers were normalized to the 16S rRNA gene copy number in each sample. Data are represented as the mean ± SEM (n ≥ 6 in each group). *P<0.05, **P<0.01. C = Control; P = Placebo; T = TUDCA; U = UDCA; G = GUDCA.

Figure 4. Orally administered UDCA, TUDCA and GUDCA undergo extensive biotransformation. Fecal samples were collected at day 4 of colitis and bile acids were quantified using UHPLC-HRMS. (A) UDCA, (B) TUDCA, (C) LCA and (D) GUDCA. Data are
represented as the mean ± SEM (n ≥ 7 in each group). *P<0.05, **P<0.01. C = Control; P = Placebo; T = TUDCA; U = UDCA; G = GUDCA.
Figure 1

A. % body weight change over time with different treatment groups.

B. DAI score changes over time with different treatment groups.

C. Colon length changes over time with different treatment groups.

D. Colonic CXCL1 and G-CSF levels with different treatment groups.

E. Serum CXCL1 and G-CSF levels with different treatment groups.
Figure 2

A. Species richness

B. Species diversity

C. Bacterial load

D. F/B ratio

E. Relative abundance (%)

- Unclassified Bacteria
- Candidatus Saccharibacteria
- Deferribacteres
- Proteobacteria
- Firmicutes
- Bacteroidetes
Figure 3

A. **Bacteroidaceae**

B. **Porphyromonadaceae**

C. **Prevotellaceae**

D. **Clostridium cluster XIVa**

E. **Akkermansia**

F. **A. muciniphila**

G. **Unclassified Bacteroidetes**

Legend:
- Unclassified Bacteria
- Verrucomicrobiae
- Saccharibacteria_incertae_sedis
- Deferrribacteres
- Unclassified Proteobacteria
- Gammaproteobacteria
- Betaproteobacteria
- Unassigned Firmicutes
- Bacilli
- Erysipelotrichia
- Clostridia
- Unclassified Bacteroidetes
- Bacteroidia

Y-axis: Relative abundance (%)
X-axis: C, P, T, U, G, No DSS, DSS
Figure 4

A. UDCA
B. TUDCA
C. LCA
D. GUDCA

ng/mg feces

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* indicates significance levels.