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Article

Comparative effect of ciprofloxacin and moxifloxacin on the modulation of bile acid profiles and gut microbiota in rats

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Fluoroquinolones are an important class of antimicrobial agents to manage infectious diseases. However, knowledge about how host bile acids are modified by fluoroquinolones is limited. We investigated and compared the impact of fluoroquinolones on circulating bile acid profiles and gut microbiota from *in vivo* studies. We administered ciprofloxacin (100 mg/kg/day) or moxifloxacin (40 mg/kg/day) orally to male Wistar rats for seven days. Fifteen bile acids (BAs) from the serum and large intestine were quantified by HPLC-MS/MS. The diversity of gut microbiota after ciprofloxacin and moxifloxacin treatment was analyzed using high-throughput, next-generation sequencing technology. The two fluoroquinolone-treated groups had different BA profiles. Ciprofloxacin significantly reduced the hydrophobicity index of the BA pool, reduced secondary BAs, and increased taurine-conjugated primary BAs in both the serum and large intestine as compared with moxifloxacin. Besides, ciprofloxacin treatment altered intestinal microbiota with a remarkable increase in *Firmicutes* to *Bacteroidetes* ratio, while moxifloxacin exerted no effect. What we found suggests that different fluoroquinolones have a distinct effect on the host BAs metabolism and intestinal bacteria, and therefore provide guidance on the selection of fluoroquinolones to treat infectious diseases.

Keywords: Ciprofloxacin. Moxifloxacin. Bile acids. Hydrophobicity index. Gut microbiota.

INTRODUCTION

Bile acids (BAs) are amphipathic biological detergents whose primary function is lipid metabolism, but they also have a wide range of regulatory functions throughout the body (Chiang, 2009). Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary BAs synthesized in the liver (Russell, 2003). They are conjugated with either glycine or taurine and stored in the gallbladder (He, Bames, Falany, 2003). BAs are then secreted into the gastrointestinal tract, where they are subsequently deconjugated, dehydroxylated, and oxidized in the intestinal lumen by gut microbes to generate the hydrophobic secondary BAs: deoxycholic acid (DCA) and lithocholic acid (LCA) (Chiang, Ferrell, 2018; Ridlon, Kang, Hylemon, 2014). BAs are critical contributors to cholesterol metabolism, lipid digestion, host-microbe interactions, and regulatory pathways in humans (Chiang, 2009; Ridlon, Kang, Hylemon, 2006). The composition of the bile acid pool is important not only to BA signaling pathways but also to BA-induced toxicity. Many different subtypes of BAs are distributed throughout

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the body; their chemical composition differs widely and they all impact health.Oral antibiotics have been used in animal models recently to explore BA metabolism. Antibiotics can alter the intestinal bacterial composition and thus might affect BA metabolism (Li *et al*., 2017; Zhang *et al*., 2014). Moreover, antibiotics may affect transporters located in the liver and gastrointestinal tract and consequently change BA enterohepatic circulation (Miyata *et al*., 2015). However, knowledge about how host BAs are modified by antibiotics is still limited. Disturbing the BA pool by temporary antibiotic-induced dysbiosis may result in a variety of disease states. For example, the fact that antibiotics alter the BA profiles may be the reason why they cause diarrhea or changes in lipid and glucose metabolism (Hashimoto *et al*., 1996). Therefore, an in-depth study of the changes in BA profiles by antibiotics is important for the rational use of antibiotics.The fluoroquinolones generally demonstrate potent activity against Gram-negative, Gram-positive, and atypical pathogens associated with infections involving the upper and lower respiratory tracts, the skin and soft tissue, and the genitourinary tract (Smith, Lomaestro, 2003). The common side effects of fluoroquinolones are gastrointestinal disturbances, headaches, skin rashes, and allergic reactions. The extensive use of fluoroquinolones has also led to concern for potential blood glucose fluctuation (Aspinall *et al*., 2009; Chou *et al*., 2013). Although fluoroquinolones share many common characteristics, they differ in their pharmacokinetic properties, spectra of activity, and safety profiles. The most common side effects of ciprofloxacin are related to the gastrointestinal tract; ciprofloxacin is a ligand of GP-BAR1, a cell surface bile acid-activated receptor highly expressed in the ileum and colon (Cipriani *et al*., 2011). However, the effect of fluoroquinolones on host BAs is not fully understood. Before we illustrate the effect of fluoroquinolones on human bile acid profiles, we first examine this effect in rats. Ciprofloxacin and moxifloxacin are commonly prescribed fluoroquinolones. In the present study, they were selected to systematically explore the impact of fluoroquinolones on circulating BA profiles and gut microbiota from *in vivo* studies and to compare their impact on BAs.

MATERIAL AND METHODS

Material

Ciprofloxacin and moxifloxacin were purchased from J&K Scientific Ltd. (Shanghai, China). CA, purity ≥ 98%), chenodeoxycholic acid (CDCA, purity ≥ 97%), ursodeoxycholic acid (UDCA, purity ≥ 99%), deoxycholic acid (DCA, purity ≥ 98%), lithocholic acid (LCA, purity ≥ 95%), tauro-cholic acid (TCA, purity ≥ 98%), tauro-chenodeoxycholic acid (TCDCA, purity ≥ 97%), tauro-ursodeoxycholic acid (TUDCA, purity ≥ 95%), tauro-deoxycholic acid (TDCA, purity ≥ 98%), tauro-lithocholic acid (TLCA, purity ≥ 98%), glycocholic acid (GCA, purity ≥ 97%), glycochenodeoxycholic acid (GCDCA, purity ≥ 97%), glycoursodeoxycholic acid (GUDCA, purity ≥ 96%), glycodeoxycholic acid (GDCA, purity ≥ 97%), and lithocholic acid-2,2,3,4,4-d5 (D5-LCA, purity 98%, internal standard (IS)) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycolithocholic acid (GLCA, purity ≥ 98%) was purchased from J&K Scientific Ltd. (Shanghai, China). Pentobarbital was also bought from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatment

Male Wistar rats (180–220 g), purchased from SLAC Laboratory Animal Ltd. (Shanghai, China), were housed in a room under controlled humidity (50% ± 5%) and temperature (23°C ± 1°C) with a 12 h light/dark cycle. The animals were fed a commercial stock diet and water *ad libitum*. All animal maintenance and treatment were approved by the Animals Ethics Committee of Suzhou Institute for Drug Control and conducted in accordance with the Clauses and General Recommendation of the Chinese Experimental Animal Administration Legislation.

Rats were acclimated for one week before starting treatment. Normal rats were randomly divided into three groups. Rats in the control group received only vehicle (0.25% of carboxymethyl cellulose solution). The other two groups were the ciprofloxacin treatment group (CFLX) and moxifloxacin treatment group (MFLX), and these rats received 100 mg/kg/day ciprofloxacin and 40 mg/kg/day of moxifloxacin orally for seven days, respectively.

Sample collection

The rats were fasted overnight before surgery and anesthetized with 60 mg/kg sodium pentobarbital injected intraperitoneally. The cephalic artery was cannulated, and the blood was collected. Serum samples obtained were stored at −80°C for assessing BAs. Then, the contents of the large intestine were collected and immediately stored at −80°C until further analysis for BAs and intestinal bacteria.

LC-MS/MS analysis of BAs

Sample preparation

For the serum samples, 400 µL of acetonitrile was added to 200 µL of serum spiked with 10 µL of IS, vortexed, and centrifuged at 20000 × g for 10 min. The supernatant was aspirated, evaporated under vacuum, and reconstituted in 100 µL of 50% methanol.For the intestine content samples, approximately 1 g of content was homogenized in 5 volumes (5 mL) of deionized water and centrifuged at 3000 × g for 5 min. 200 µL of acetonitrile and 10 µL of 14% ammonium hydroxide were added to 200 µL of supernatant spiked with 10 µL of IS. The mixture was centrifuged at 20000 × g for 10 min. The supernatant was aspirated, evaporated under vacuum, and reconstituted in 100 µL of 50% methanol.

BA quantification in serum and large intestine

An Agilent 1260 HPLC system was interfaced with an AB Sciex 4000 MS system. Mobile phase A was 10 mmol/L ammonium acetate solution, and the mobile phase B was acetonitrile. Gradient chromatographic separation of BAs was performed on a 150 mm × 4.60 mm Xterra® RP18 column with a particle size of 5 µm. The elution gradient started with 30% B for 16 min, increased to 90% until 18 min, and held for 5 min, followed by a 6.5 min decrease to 30% B starting at 23.5 min. The flow rate was 1.0 mL/min. The MS turbo ion spray source was operated in negative ion mode using the following settings: ion spray voltage = −4200 V; ion source heater temperature = 500°C; source gas 1 = 50 psi; source gas 2 = 50 psi; curtain gas = 30 psi. BAs were monitored by multiple-reaction monitoring. Mass transitions and MS parameters are described in Supplementary Table SI. Quadrupoles Q1 and Q3 were operated at unit resolution. The lower limit of quantification (LLOQ) for all BAs was 1 ng/mL. Subgrouping of the measured BAs are listed in Table I. The calibration curves of the BAs were linear in the quantitative range. Endogenous substances in the blank matrix did not interfere with the detection of BA components and the IS. The relative standard deviations of the intra-day and inter-day precision results were all less than 15%. The average extraction recoveries of the control samples in the serum and intestinal content homogenate were 69.93%–88.83% and 83.16%–109.3%, respectively. The matrix effects ranged from 85.41% to 113.57% in the serum and from 94.64% to 112.86% in the intestinal content homogenate. All BAs were stable for 24 hours at room temperature, 72 hours at 4°C, three months at −80°C, and to freeze-thaw cycles.

TABLE I - Subgrouping of the measured BAs

Name	Primary	Secondary	Conjugated
CA	√		
CDCA	√		
UDCA			
DCA		√	
LCA		√	
TCA	√		√
TCDCA	√		√
TUDCA			√
TDCA		√	√
TLCA		√	√
GCA	√		√
GCDCA	√		√
GUDCA			√
GDCA		√	√
GLCA		√	√

Gut microbiota determination by 16S rRNA sequencing

Bacterial DNA extraction

Total genome DNA was extracted from 250 mg of intestinal contents using a commercial DNA extraction kit (Tiangen Biotech Corporation, Beijing, China) following the manufacturer's protocol and quantified using a Qubit 2.0 fluorometer (Invitrogen Corporation, Carlsbad, CA, USA). The concentration and purity of the total DNA were assessed by 0.8% agarose gel electrophoresis and spectrophotometry (optical density at 260/280 nm). All extracted DNA samples were stored at −80°C until further analysis.

Library preparation and Illumine MiSeq sequencing

Next-generation sequencing library preparations and Illumina MiSeq sequencing were conducted by GENEWIZ, Inc. (Suzhou, China). A library sequence of the V3 and V4 regions of 16S rDNA was constructed using a 30–50 ng DNA aliquot isolated from each fecal sample. The V3 and V4 regions were amplified by polymerase chain reaction (PCR) using the following primer pair: forward 5′–CCTACGGRRBGCASCAGKVRV GAAT–3′ and reverse 5′–GGACTACNYVGGGTWTCTAATCC–3′. PCR reactions were performed in triplicate using a 25 μL mixture containing 2.5 μL of TransStart Buffer, 2 μL of dNTPs, 1 μL of each primer, and 20 ng of template DNA. The first-round PCR products were used as templates for a second round of amplicon enrichment by PCR (94°C for 3 min, followed by 24 cycles at 94°C for 5 s, 57°C for 90 s, 72°C for 10 s, and a final extension at 72°C for 5 min). Indexed adapters were added to the ends of the 16S rDNA amplicons to generate the indexed libraries that were ready for downstream NGS on the MiSeq platform. DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified with a Qubit 2.0 Fluorometer. DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing was performed using a 2 × 300 paired-end configuration. Image analysis and base calling were conducted with the MiSeq Control Software provided with the MiSeq instrument. The sequences generated here were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the accession numbers SAMN12205734-12205745.

16S rRNA sequencing analysis

16S rRNA data were analyzed using the QIIME data analysis package. The forward and reverse reads were joined and assigned to samples based on their barcode and truncated by cutting off the barcode and primer sequence. Quality filtering was performed on joined sequences and sequence, which did not fulfill the following criteria were discarded: sequence length < 200 bp, ambiguous bases, mean quality score ≥ 20. The sequences were then compared with the reference database [the Ribosomal Database Project Ribosomal Database Program (RDP) Gold database] using the UCHIME algorithm to detect chimeric sequences, and the chimeric sequences were removed.The effective sequences were grouped into operational taxonomic units using the clustering program VSEARCH (1.9.6) against the Silva 132 database pre-clustered at 97% sequence identity. The RDP classifier was used to assign a taxonomic category to all operational taxonomic units with a confidence threshold of 0.8. The RDP classifier uses the Silva 132 database (http://www.arb-silva.de/), which has taxonomic categories predicted to the species level.

Statistical analysis

BA values below the LLOQ were replaced by the LLOQ limit. The results are expressed as the mean ± standard error (S.E.M). Data were statistically analyzed by performing two-tailed non-paired t-tests. A p-value of less than 0.05 was considered statistically significant. The heatmaps of BAs and intestinal bacteria were generated using HemI 1.0.3.7.

RESULTS AND DISCUSSION

Effect of ciprofloxacin and moxifloxacin on serum BA composition

The amount of serum BAs in ciprofloxacin- and moxifloxacin-treated rats was reduced to approximately 15% and 40% of that in untreated normal rats, respectively (Figure 1A). Ciprofloxacin treatment markedly increased total taurine-conjugated primary BAs [TCA (349% ↑) and TCDCA (77% ↑)], while moxifloxacin treatment did not significantly impact TCA or TCDCA. Both fluoroquinolones decreased secondary BAs. Ciprofloxacin significantly decreased LCA (98% ↓) and DCA (99% ↓) and moxifloxacin decreased LCA (70% ↓) and DCA (61% ↓). The heatmap of serum BAs is shown in Figure 2 and the concentrations of 15 BAs in serum are shown in Supplementary Table SII.The exact contribution of each BA to the whole body was difficult to appreciate since each BA can bind to and modulate the activity of transmembrane and nuclear receptors (Kundu, Kumar, Bajaj, 2015). The hydrophobicity index (HI) of the circulating BA profiles quantitatively defines the composite hydrophilic-hydrophobic balance of a mixture of BAs. We calculated the HI of the circulating BA pool after ciprofloxacin and moxifloxacin treatment and calculated the concentration of individual BA components using the formula (where HIx is the HI of pure bile salt and Fx is the mole fraction of biliary bile salts) as previously described (Heuman, 1989). Ciprofloxacin significantly decreased the HI of serum BAs in normal rats, while moxifloxacin did not significantly impact it (Table II).

The extensive use of fluoroquinolones may cause potential blood glucose fluctuation (Aspinall *et al.*, 2009; Chou *et al.*, 2013). The risk of a clinically relevant dysglycemic event varies between fluoroquinolones (Aspinall *et al.*, 2009; Ghaly *et al.*, 2009). Patients with type 2 diabetes had elevated serum BA HI due to the higher circulating levels of DCA and its conjugated forms (Haeusler *et al.*, 2013). Serum taurine-conjugated BAs also decreased in obese and diabetic patients (Jeevanandam, Ramias, Schiller, 1991; Tsuboyama-Kasaoka *et al.*, 2006). Besides, taurine controls glucose homeostasis and islet function (Carneiro *et al.*, 2009). Therefore, our finding that ciprofloxacin significantly reduces HI and increases taurine-conjugated BAs reveal a potential hypoglycemic effect of ciprofloxacin on glucose homeostasis.

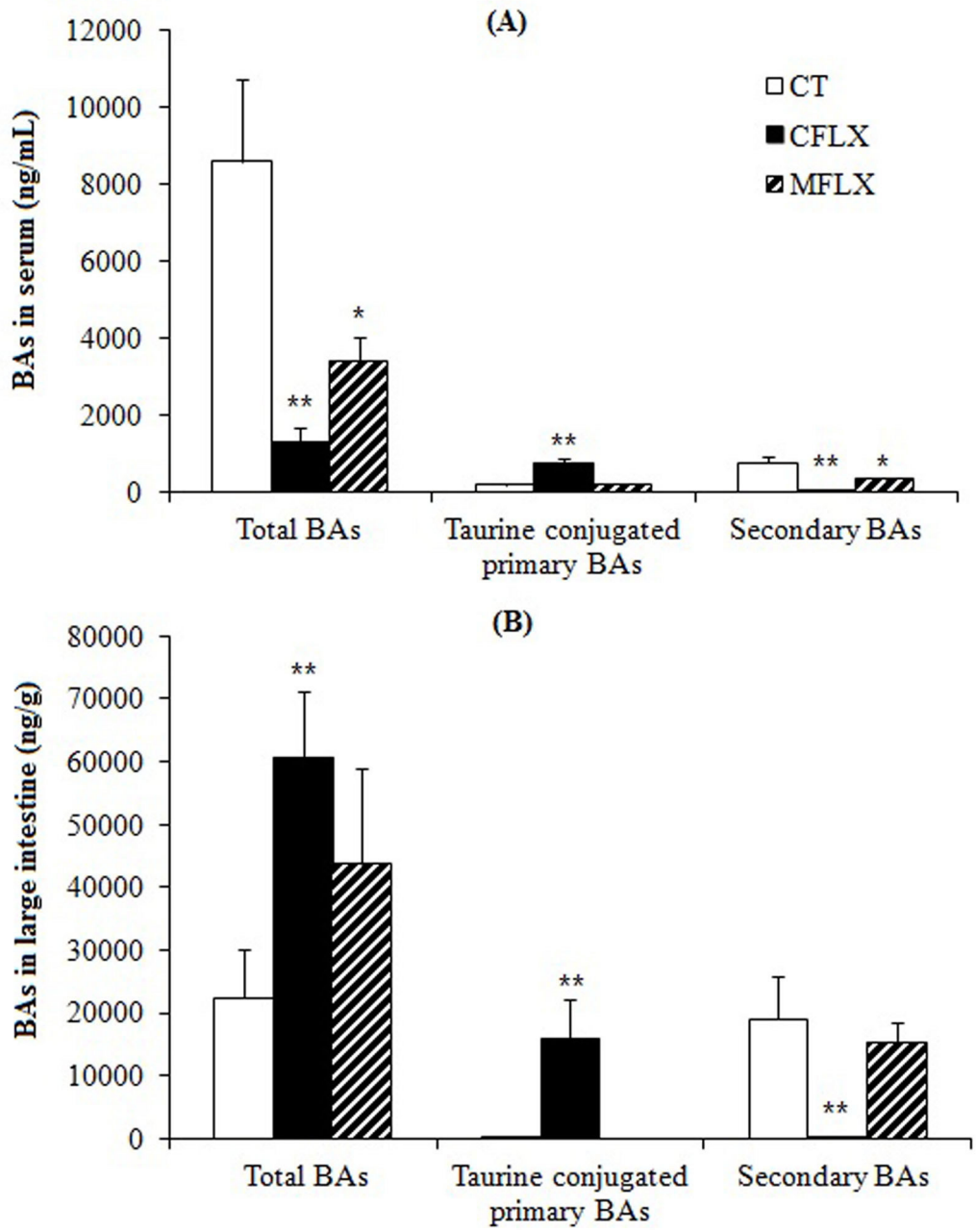

FIGURE 1 - Total BAs, taurine-conjugated primary BAs, and secondary BAs concentrations in serum and large intestine of normal rats treated with 100 mg/kg/day ciprofloxacin and 40 mg/kg/day of moxifloxacin orally for seven days. Data are expressed as the mean ± S.E.M (n = 5). *$p < 0.05$, **$p < 0.01$ vs. CT rats.

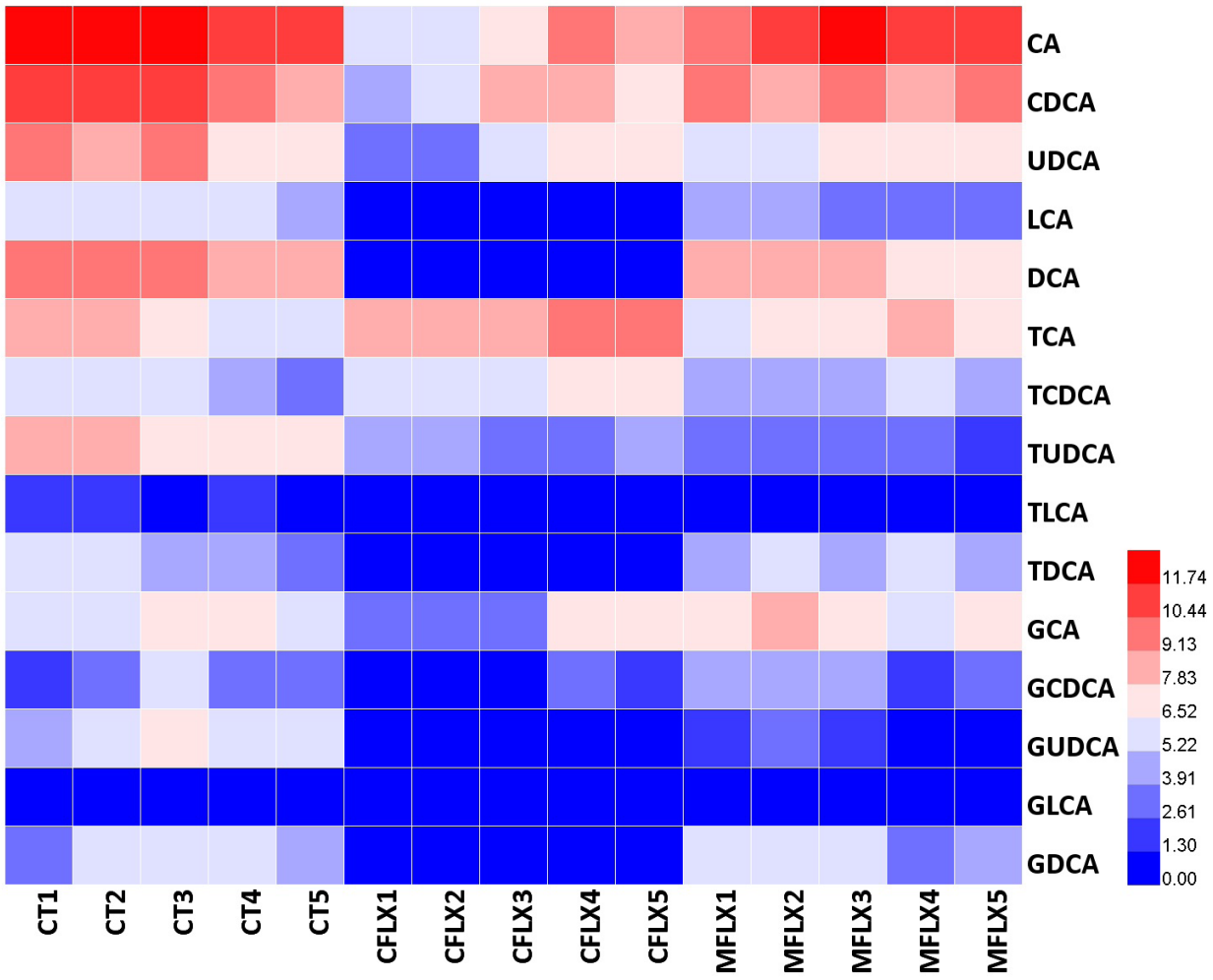

FIGURE 2 - Heatmap of the effect of ciprofloxacin and moxifloxacin on host BA profiles in serum of normal rats treated with 100 mg/kg/day ciprofloxacin and 40 mg/kg/day of moxifloxacin orally for seven days. The heatmap was generated using HemI 1.0.3.7. Red indicates an high value, whereas blue indicates a low value.

TABLE II - Effect of ciprofloxacin and moxifloxacin on HI of bile acids pool

HI	CT	CFLX	MFLX
Serum	0.235 ± 0.027	0.120 ± 0.052**	0.247 ± 0.014
Large intestine	0.545 ± 0.028	0.097 ± 0.009**	0.366 ± 0.062*

Data are means ± S.E.M (n = 5)
*$p < 0.05$ and **$p < 0.01$ compared to CT rats

Effect of ciprofloxacin and moxifloxacin on BA composition in large intestine

Contents of the large intestine were collected and BAs were quantified to evaluate the effect of ciprofloxacin and moxifloxacin on BA metabolism in the intestine. As shown in Figure 1B, ciprofloxacin and moxifloxacin slightly increased total BAs in large intestines. However, their effects on taurine-conjugated primary BAs and secondary BAs were different. Ciprofloxacin significantly increased taurine-conjugated primary BAs, including TCA (40000% ↑) and TCDCA (13600% ↑), whereas moxifloxacin decreased taurine-conjugated primary BAs [TCA (30% ↓) and TCDCA (84% ↓)]. Ciprofloxacin

markedly reduced secondary BAs, including LCA (99% ↓) and DCA (99% ↓) while moxifloxacin did not significantly affect them. The heatmap of BAs in the large intestine is shown in Figure 3 and the concentrations of 15 BAs in the large intestine are shown in Supplementary Table SIII.

TABLE III - Effect of ciprofloxacin and moxifloxacin on TCA/DCA and TCDCA/LCA in intestine

Ratios	CT	CFLX	MFLX
TCA/DCA	0.003 ± 0.001	600 ± 145*	0.002 ± 0.001
TCDCA/LCA	0.004 ± 0.002	66.3 ± 15.4*	0.003 ± 0.001

Data are means ± S.E.M (n = 5)
*$p < 0.05$ compared to CT rats

Since DCA is the metabolic product of TCA and LCA is the metabolic product of TCDCA, we calculated the TCA/DCA and TCDCA/LCA ratios. We found that moxifloxacin did not affect either ratio, whereas ciprofloxacin remarkably increased both in the large intestine (Table III, $p < 0.05$). Consequently, ciprofloxacin significantly decreased the HI of BAs in the large intestine (Table II, $p < 0.01$). This indicated that ciprofloxacin markedly inhibited intestinal secondary BA formation.

The present study suggests that ciprofloxacin may protect the liver and intestine from injuries induced by toxic BAs. Extensive researches strongly suggest that secondary BAs are involved in many human gastric and hepatic diseases. Increased production of DCA is associated with the formation of hepatocellular carcinomas and intestinal cancers (Dong *et al.*, 2018; Yoshimoto *et al.*, 2013). LCA is implicated in intrahepatic cholestasis (Alkhedaide *et al.*, 2018; Lucangioli *et al.*, 2009). The toxicity of BAs is partially attributed to their detergent properties, which is dependent on their hydrophilicity. Ciprofloxacin significantly increased the hydrophilicity of BAs in both the serum and intestine, especially by remarkably decreasing hydrophobic secondary BAs DCA and LCA concentration.

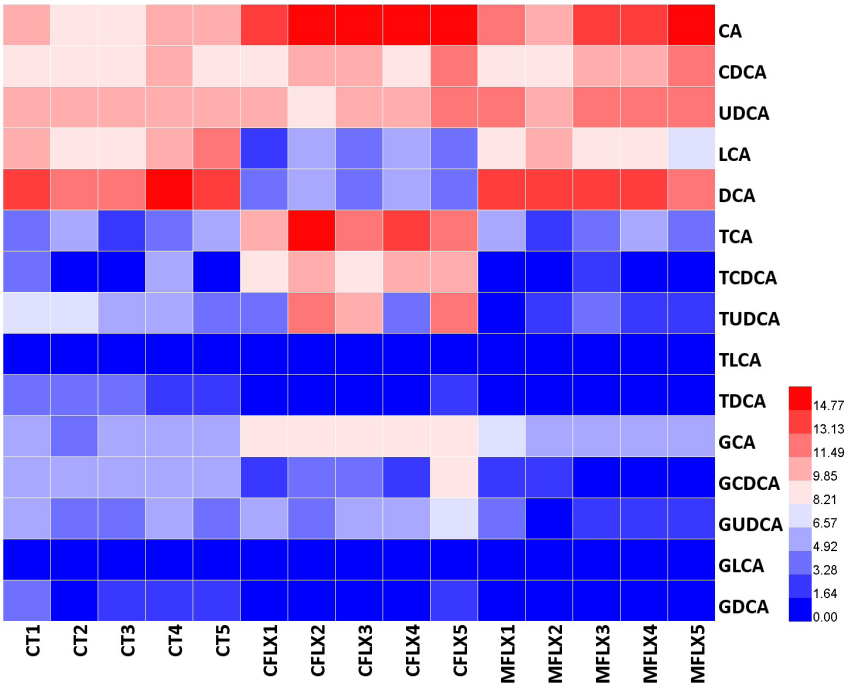

FIGURE 3 - Heatmap of the effect of ciprofloxacin and moxifloxacin on host BA profiles in the large intestine of normal rats treated with 100 mg/kg/day ciprofloxacin and 40 mg/kg/day of moxifloxacin orally for seven days. The heatmap was generated using HemI 1.0.3.7. Red indicates an increased value, whereas blue indicates a decreased value.

Effect of ciprofloxacin and moxifloxacin on intestinal microbiota

Antibiotics ultimately change the composition and concentration of BAs mainly by altering the gut microbiota (Antunes *et al.*, 2011; Sayin *et al.*, 2013). The intestinal microbiota in both humans and rats consists mainly of species belonging to the *Firmicutes* and *Bacteroidetes* phyla (Ley *et al.*, 2008; Salzman *et al.*, 2002). We quantified 42 individual bacteria in the intestinal tract after ciprofloxacin and moxifloxacin treatment, among which 25 were *Firmicutes* and 12 were *Bacteroidetes*. We found that ciprofloxacin significantly increased two main *Firmicutes* [*Lactobacillus* (200% ↑) and *Lachnoclostridium* (1200% ↑)]. By contrast, moxifloxacin suppressed *Lactobacillus* (54% ↓) and *Lachnoclostridium* (95% ↓). Other *Firmicutes* were almost totally suppressed by both ciprofloxacin and moxifloxacin. Ciprofloxacin significantly suppressed all the *Bacteroidetes* we detected. Moxifloxaicn had a weaker effect on *Bacteroidetes* than ciprofloxacin. It is worth noting that ciprofloxacin significantly reduced *Bacteroides* (99% ↓) whereas moxifloxacin increased *Bacteroides* (128% ↑). The heatmap of intestinal bacteria is shown in Figure 4 and the genus abundance of individual bacteria is shown in Supplementary Table SIV.

In general terms, the *Firmicutes* to *Bacteroidetes* ratio is relevant to the composition of intestinal microbiota (Zhang *et al.*, 2014). Here, we found that ciprofloxacin significantly increased the *Firmicutes* to *Bacteroidetes* ratio mainly due to the increase of *Lactobacillus* and *Lachnoclostridium* and decrease of *Bacteroidetes* (Table IV, $p < 0.01$). Unlike ciprofloxacin, moxifloxacin did not affect the *Firmicutes* to *Bacteroidetes* ratio (Table IV).

Secondary BAs are generated in the distal intestinal lumen by gut microbes from primary BAs, which are secreted into the gastrointestinal tract by the gallbladder (Sagar *et al.*, 2015). Many of the intestinal bacteria, especially some of the *Bacteroides* and *Clostridia*, are active in the transformation of primary to secondary BAs (Sagar *et al.*, 2015; Staley *et al.*, 2017). We showed that ciprofloxacin significantly reduced *Bacteroides* but moxifloxacin increased them. Therefore, a positive correlation exists between intestinal secondary BAs and intestinal *Bacteroides*, which may explain why ciprofloxacin but not moxifloxacin inhibited secondary BAs formation in the intestine.

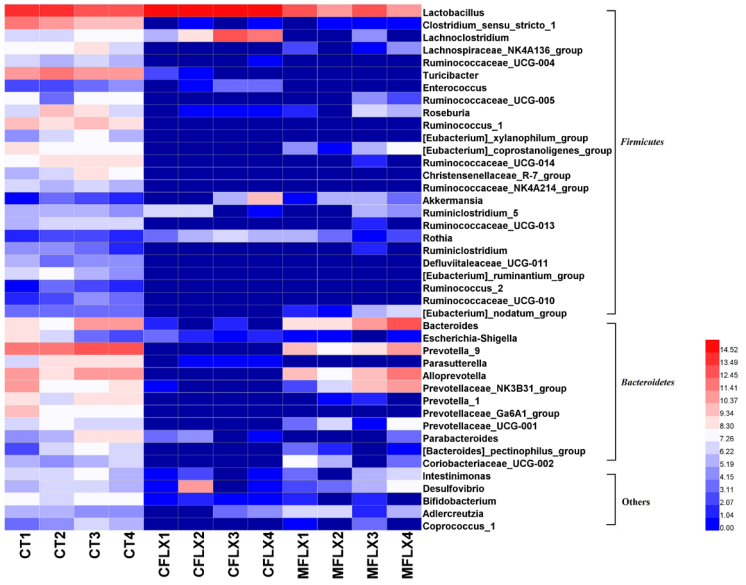

FIGURE 4 - Heatmap of ciprofloxacin and moxifloxacin modulation on the gut microbiota of normal rats treated with 100 mg/kg/day ciprofloxacin and 40 mg/kg/day of moxifloxacin orally for seven days. The heatmap was generated using HemI 1.0.3.7. Red indicates an increased value, whereas blue indicates a decreased value.

TABLE IV - Effect of ciprofloxacin and moxifloxacin on *Firmicutes*/*Bacteroidetes* ratio in large intestine

Groups	*Firmicutes*/*Bacteroidetes*
CT	2.142 ± 0.7201
CFLX	3825 ± 1392**
MFLX	1.963 ± 0.7021

Data are means ± S.E.M (n = 4)
**$p < 0.01$ compared to CT rats

CONCLUSION

We demonstrated that fluoroquinolone treatment markedly changed intestinal bacterial communities and diversity and systemically affected BA profiles. Ciprofloxacin was more efficient in suppressing secondary BA formation in the intestine, resulting in increased conjugated primary BAs in serum and intestine of rats. However, the physiopathological significance of the BA profile changes induced by ciprofloxacin requires further investigation. Overall, our findings will help select fluoroquinolones for infectious disease treatment.

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SUPPLEMENTARY INFORMATION

See Supplementary Tables SI-SIV in the Supplementary Information.

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SUPPLEMENTARY TABLES

Supplementary Table SI - Summary of bile acids and Mass Spectrometry conditions

Retention Time (min)	Precursor Ion (Da)	Product Ion (Da)	Dwell Time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
4.66	407.40	407.40	100	-177	-10	-18	-11
13.40	391.41	391.41	100	-174	-10	-14	-10
6.37	391.40	391.30	100	-170	-10	-12	-11
14.60	391.40	391.40	100	-180	-10	-13	-10
21.04	375.10	375.10	100	-200	-10	-12	-10
4.43	514.50	514.50	100	-220	-10	-13	-14
10.80	498.40	498.40	100	-270	-10	-8.4	-14
4.95	482.20	482.40	100	-200	-10	-9.5	-14
12.80	498.50	498.50	100	-220	-10	-20	-14
19.39	482.30	482.30	100	-206	-10	-29	-11
3.75	464.40	464.40	100	-145	-10	-10	-10
8.22	448.40	448.20	100	-150	-10	-10	-12
3.97	448.40	448.40	100	-150	-10	-13	-10
10.00	448.50	448.50	100	-150	-10	-24	-10
19.30	432.50	432.30	100	-150	-10	-12	-11
21.00	380.40	380.40	100	-200	-9	-12	-8

Supplementary Table SII - Concentrations of 15 BAs in serum of rats

BAs ($ng \cdot mL^{-1}$)	CT	CFLX	MFLX
CA	4950±1246	279.3±126.2*	2000±506.5
CDCA	1853±540.5	158.4±52.69*	567.6±93.06
UDCA	438.2±125.1	58.80±22.26	125.1±30.11
DCA	614.4±132.7	ND**	237.2±32.47*
LCA	56.60±9.722	ND**	16.58±5.714*
TCA	143.6±41.35	645.0±151.4	163.0±31.92
TCDCA	43.14±13.18	76.24±15.69	33.68±3.613
TUDCA	220.9±74.12	17.94±2.547*	9.222±0.9852*
TDCA	36.66±13.99	ND*	29.24±3.671
TLCA	2.384±0.3148	ND*	1.482±0.1289*

Supplementary Table SII - Concentrations of 15 BAs in serum of rats

BAs ($ng \cdot mL^{-1}$)	CT	CFLX	MFLX
GCA	103.8±30.74	59.41±31.25	159.3±42.29
GCDCA	17.19±8.094	3.150±1.640	13.24±2.667
GUDCA	86.06±28.56	ND*	4.068±1.154*
GDCA	44.52±12.67	ND*	38.30±10.18
GLCA	1.204±0.1415	ND	ND

Data are means ± S.E.M (n=5)
*p<0.05 and **p<0.01 compared to CT rats

Supplementary Table SIII - Concentrations of 15 BAs in large intestine of rats

BAs ($ng \cdot g^{-1}$)	CT	CFLX	MFLX
CA	1380±403.4	38160±6047**	23047±16177
CDCA	701.1±214.4	2004±1050	1782±741.7
UDCA	1517±276.2	1697±634.5	3526±369.8**
DCA	16659±6426	22.37±5.761	14214±2953
LCA	2280±751.0	22.55±5.660*	867.6±374.8
TCA	36.20±11.22	14537±5991*	25.02±9.489
TCDCA	10.23±6.893	1403±356.4*	1.600±0.6000
TUDCA	73.900±20.467	2183.8±980.66	7.0200±2.139
TDCA	9.820±1.584	2.730±1.310	1.280±0.2800
TLCA	1.111±0.06929	1.4360±0.2647	1.1690±0.1086
GCA	43.48±9.166	503.2±82.10**	100.1±22.13
GCDCA	70.10±2.015	96.69±84.42	3.700±1.288**
GUDCA	32.51±9.153	67.01±31.69	6.340±1.169*
GDCA	9.430±4.1193	1.670±0.6700	1.410±0.2331
GLCA	ND	ND	ND

Data are means ± S.E.M (n=5)
*p<0.05 and **p<0.01 compared to CT rats

Supplementary Table SIV - The genus abundance of intestinal microbiome

Intestinal microbiome	CT	CFLX	MFLX
Lactobacillus	12234±2300	37010±4891**	5612±2173
Clostridium_sensu_stricto_1	1738±484.4	0.7500±0.4787*	1.250±0.4787*
Lachnoclostridium	179.3±46.41	2372±1343	8.750±8.750*
Lachnospiraceae_NK4A136_group	216.5±63.00	ND*	9.750±7.530*
Ruminococcaceae_UCG-004	92.25±18.44	0.25±0.25**	ND**
Turicibacter	2584±668.7	2.250±1.650**	ND**
Enterococcus	15.50±4.970	7.000±3.760	ND*
Ruminococcaceae_UCG-005	190.8±61.51	ND*	8.250±6.140*
Roseburia	350.8±145.7	0.7500±0.2500	41.00±22.77
Ruminococcus_1	825.3±189.0	ND**	ND**
[Eubacterium]_xylanophilum_group	89.25±33.13	ND*	ND*
[Eubacterium]_coprostanoligenes_group	269.3±46.65	ND**	63.00±36.20*
Ruminococcaceae_UCG-014	425.3±47.86	ND**	1.000±1.000**
Christensenellaceae_R-7_group	226.3±81.32	ND	ND
Ruminococcaceae_NK4A214_group	62.75±11.67	ND*	ND*
Akkermansia	5.250±2.170	238.3±225.1	24.00±9.890
Ruminiclostridium_5	40.75±6.96	47.00±26.56	18.00±10.52
Ruminococcaceae_UCG-013	96.00±10.30	ND**	0.75±0.75**
Rothia	4.750±0.8500	55.00±16.85*	14.00±8.220
Ruminiclostridium	13.25±3.450	ND**	0.7500±0.7500*
Defluviitaleaceae_UCG-011	27.75±8.2600	ND*	ND*
[Eubacterium]_ruminantium_group	91.50±40.51	ND	ND
Ruminococcus_2	5.000±1.470	ND*	ND*
Ruminococcaceae_UCG-010	11.00±5.120	ND	ND
[Eubacterium]_nodatum_group	12.50±1.320	ND**	37.75±24.54
Bacteroides	999.5±418.6	2.000±1.150*	2283.5±1464.2
Escherichia-Shigella	162.5±116.9	5.250±2.660	0.7500±0.2500
Prevotella_9	5830±1065.3	ND**	864.8±413.6**
Parasutterella	346.5±101.9	1.00±0.41*	ND*
Alloprevotella	1567±425.5	ND*	1340.50±906.00
Prevotellaceae_NK3B31_group	854.5±540.7	0.2500±0.2500	665.8±403.6
Prevotella_1	361.0±104.1	ND*	1.000±0.7100*
Prevotellaceae_Ga6A1_group	344.8±168.21	ND	ND

Supplementary Table SIV - The genus abundance of intestinal microbiome

Intestinal microbiome	CT	CFLX	MFLX
Prevotellaceae_UCG-001	104.8±18.64	ND**	81.00±49.09
Parabacteroides	263.3±123.4	8.50±4.73	3.750±3.750
[Bacteroides]_pectinophilus_group	137.8±63.00	ND	4.250±2.720
Coriobacteriaceae_UCG-002	73.00±26.52	ND*	70.50±52.37
Intestinimonas	105.0±23.28	2.250±1.310**	32.75±17.25*
Desulfovibrio	74.75±21.94	459.8±458.4	63.00±40.52
Bifidobacterium	198.5±33.33	1.50±0.50**	1.750±1.030**
Adlercreutzia	35.75±9.200	7.000±4.530*	68.00±29.36
Coprococcus_1	43.00±18.36	ND	4.000±3.670

Data are means ± S.E.M (n=4)
*p<0.05 and **p<0.01 compared to CT rats