Regulatory Toxicology and Pharmacology **33,** 276–284 (2001)
doi:10.1006/rtph.2001.1473, available online at http://www.idealibrary.com on IDEAL®

Effects of Low Levels of Ciprofloxacin on a Chemostat Model of the Human Colonic Microflora

Robert J. Carman and Mary Alice Woodburn

TechLab, Inc., 1861 Pratt Drive, Blacksburg, Virginia 24060-6364

Received April 13, 2000

To study the utility of an *in vitro* model system for assessing the effect of low concentrations of a fluoroquinolone (FQ) drug on the ecology of the human intestinal microflora, chemostats containing human fecal flora were exposed to 0.43, 4.3, and 43 μg of ciprofloxacin (CI) per milliliter. Prior to and during drug exposure, we assayed short-chain fatty acids (SCFA), bacterial populations, and the relative levels of susceptibility of these populations to CI and trovafloxacin (TV), a newer related FQ with increased activity against anaerobes. The degree to which CI affected the chemostat ecology was measured statistically by comparing observed data with the corresponding predicted "no effect" level. No changes in total SCFA were observed; only butyrate was significantly higher at the intermediate and high-dose levels. Enterococci counts and the levels of susceptibility to CI among enterococci were also unaffected. *Escherichia coli* counts decreased in a dose-dependent manner. Susceptibility levels in *E. coli* followed no interpretable pattern. *Bacteroides fragilis* group (BfG) counts decreased significantly following exposure to 43 and 4.3 μg/mL CI. Ciprofloxacin susceptibility among the BfG in these chemostats was not determined because the BfG counts were too low (less than 30 colonies per plate) when undiluted chemostat samples were plated. However, within 2 days of exposure to 0.43 μg/mL CI, the percentage of BfG resistant to 4 μg/mL CI increased to over 95%. Before exposure, all BfG were susceptible to both CI (2 μg/mL) and TV (0.25 μg/mL). All BfG isolated during exposure were resistant to both CI (4 μg/mL) and TV (2 μg/mL). Resistance selection in the BfG was unexpected as the MIC_{90} of CI for *B. fragilis* is 8 μg/mL. Since the average colon flora is about 20% *B. fragilis* and other bacteroides, CI may impact the human gut flora even at subtherapeutic levels.

***Key Words*: ciprofloxacin; fluoroquinolone; microflora; human; intestinal; chemostat; drug residues.**

INTRODUCTION

The use of veterinary antimicrobial agents in food animals allows for the potential of antibiotic residues to enter the food supply and be consumed by humans. Although the impact of low levels of antibiotics on human flora remains an ill-defined hazard, the U.S. Food and Drug Administration have proposed that the acceptable daily intake (ADI) for any microbiologically active residue in edible tissues from food-producing animals should not exceed 1.5 mg/person/day (FDA, 1996). The level for some drugs may be less.

Fluoroquinolones (FQ) are therapeutic drugs used in both veterinary and human medicine. Little is known about the effects of veterinary FQ or their residues on the intestinal flora of humans. However, ciprofloxacin (CI), a human fluoroquinolone used in this study, is also an active metabolite of a veterinary FQ, enrofloxacin, residues of which may be consumed by people eating tissues from animals treated with this drug (WHO, 1995). Since enrofloxacin is partially metabolized *in vivo* to CI, the effects on the human intestinal flora of either drug may be comparable.

Ciprofloxacin is widely used in human medicine. Considerable information exists concerning its effects on the human intestinal flora at therapeutic doses (Schentag and Scully, 1999). Ciprofloxacin is intended primarily for the treatment of infections caused by aerobic and facultative anaerobes. It is not considered to be clinically effective against obligate anaerobes (Schentag and Scully, 1999). For example, the MIC_{90} of CI toward the facultative anaerobe *Escherichia coli*, a species accounting for less than 1% of the fecal flora, is 0.01–0.25 μg/mL, while for *Bacteroides fragilis* and other intestinal bacteroides it is 8 μg/mL (Schentag and Scully, 1999; Wolfson and Hooper, 1989). Since each gram of colon content contains, on average, 10^{11} bacteria, of which 20% are bacteroides and about 80% are other obligate anaerobes (Moore and Holdeman, 1974; Holdeman *et al.*, 1976), conventional wisdom

0273-2300/01 $35.00

holds that CI is unlikely to have a major impact on the human gut microflora.

The overall objective of this work was to study the utility of a chemostat system containing human fecal flora for testing the effect of CI on microbiological endpoints of the human microflora, particularly at low drug concentrations. The endpoints evaluated were population counts of target bacteria, selection of resistant bacteria, and concentrations of short-chain fatty acids (SCFA). Population counts were evaluated because they are the most common parameters studied by investigators for assessing changes in the balance of the intestinal microflora. The selection of resistant bacteria is an endpoint of high public health concern because of the possible role that the use of veterinary FQ could have on the selection of resistance to FQ used in human medicine. SCFA were evaluated because they are the most commonly studied biochemical markers of bacterial activity.

MATERIALS AND METHODS

Chemostat. A 1000-mL culture vessel was maintained under an atmosphere of nitrogen, at 37°C, and between pH 6.4 and 6.6 (pH control and dual pump model; Virtis Co. Inc., Gardiner, NY). Culture medium was pumped (101U/R peristaltic pump; Watson Marlow, Falmouth, England) into the culture vessel at a uniform rate (35 mL/h, equivalent to a dilution rate of 0.07/h). Culture medium was magnetically stirred. The volume of culture medium was 500 mL. Effluent medium passed from the chemostat to a collecting vessel via a side arm. Gas traps containing acidified silver sulfate and glycerol were used to reduce the smell of sulfide from the culture vessel and effluent carboy. The system was set up and equilibrated for 24 h before inoculation with feces.

Medium. The medium was based on a formulation of Gibson *et al.* (1988) to which physiologically relevant levels of two primary bile acids and the animal neutral sterol cholesterol were added. The medium contained (g/L deionized water): starch (5.0), bovine milk casein (3.0), peptone (3.0), pectin (0.6), xylan (0.6), arabinogalactan (0.6), amylopectin (0.6), L-cysteine (0.4), hemin (0.01), cholesterol (0.25), chenodeoxycholic acid (0.25), cholic acid (0.25), KH_2PO_4 (2.0), NaH_2CO_3 (10.0), NaCl (4.5), $MgSO_4 \cdot 7H_2O$ (0.5), and $CaCl_2 \cdot 2H_2O$ (0.45).

Fecal inoculum. Seven adult volunteers (five males, two females; 22 to 43 years old) donated their first fecal motion of the day. None of the donors had received antibiotics within the preceding 12 weeks or reported any signs of diarrhea or other intestinal tract problems during the same period. All considered themselves to be in good health and consumers of nonvegetarian diets typical of North Americans. TechLab's Institutional Review Board approved collection of feces.

Feces were passed directly into sealable plastic bags and stored at 2 to 8°C for up to 60 min inside an anaerobic chamber (Bactron IV, Sheldon Manufacturing, Inc., Cornelius, OR) with an atmosphere of 85% nitrogen, 5% hydrogen, and 10% carbon dioxide by volume. Still inside the anaerobic chamber the samples were pooled into a single bag. Glycerol was added (10% final concentration by weight) as a cryoprotectant (Guerin-Danan *et al.*, 1999). All subsequent calculations took into account the weight of the added glycerol; thus the weights of feces quoted elsewhere in this report represent feces alone, not feces and glycerol. The bag was removed from the anaerobic chamber and, while still sealed and under an anaerobic atmosphere, the fecal pool was homogenized in a stomacher (Model 3500, Seward Medical Ltd., London, England). Once the bag was returned to the chamber, aliquots of the homogenized fecal pool (approximately 11 g with the added glycerol) were dispensed into preweighed, sterile 50-mL test tubes that were stoppered in the glove box (i.e., the contents remained under an anaerobic atmosphere). The tubes were reweighed before being frozen at <−70°C and the exact weight of feces in each tube was recorded.

Feces were thawed at room temperature for approximately one hour in the anaerobic chamber. They were uniformly suspended in prereduced and anaerobically sterilized (PRAS) diluent (Randolph Biomedical, West Warwick, RI; Holdeman *et al.*, 1977) at a ratio of 1 vol of feces to 4 vol of diluent. Chemostats were inoculated with 50 mL of the suspension containing 10 g feces on days 1, 3, and 5 of the study by injection through a septum in the vessel lid.

Ciprofloxacin. Three medium concentrations of CI (Bayer Corp., West Haven, CT) were tested: 0.43, 4.3, and 43 μg/mL. A no-drug control was also run.

Ciprofloxacin was added on day 25 of chemostat operation as a concentrated solution to the autoclaved medium reservoir to achieve final reservoir medium concentrations of 0.43, 4.3, and 43 μg/ml and then pumped into the chemostat continuously. Theoretically, one complete exchange of medium would occur in the chemostat system in approximately 13.5 h; however, in practice the exchange takes longer because of the constant mixing of the medium. Accordingly, it was estimated that there was a lag period of approximately 2 days (days 25–26 inclusive) during which the concentration of drug in each chemostat rose to its nominal test level.

Bioassays were run to measure CI in the medium using *E. coli* ATCC 11775. The indicator strains were

stored at <−70°C and thawed at room temperature. Standard inocula used for the assays contained 10^6 cell/mL. One-hundred microliters of inoculum was spread onto Wilkins Chalgren agar plates. Sterile disks (6 mm diameter; Difco) of predetermined absorbency were loaded with fluid from control solutions of known concentration and from sterile medium collected between the medium reservoir and the chemostat vessel. Samples were collected for testing on 3 separate days. The disks were placed aseptically onto the agar plates and the plates were incubated overnight at 37°C. After incubation, the zones of inhibition around the disks were measured. The concentration of test compound in the medium was read from a standard curve. The assays were run in triplicate.

Time table. Preliminary studies showed that the SCFA profiles reached a steady state after 7 to 10 days of chemostat operation. Accordingly, chemostats were run for 16 days to ensure steady state. From day 17 to 24 inclusive, the medium contained no test compound. From day 25 to 32, CI was infused from the medium reservoirs as described above to attain concentrations of 0, 0.43, 4.3, and 43 μg/mL in the respective culture vessels.

Sampling. Six samples (10 mL each) were aspirated daily from each culture vessel from day 17 through day 32. Each aliquot was mixed into 1 mL of sterile glycerol that served as a cryoprotectant (Guerin-Danan *et al.*, 1999). The volume of the added glycerol was considered in all subsequent calculations. The tubes were flushed with anaerobic gasses (90% nitrogen, 10% carbon dioxide) for 5 min before freezing at <−70°C. Samples were thawed inside a glove box at room temperature for approximately 1 h prior to analysis.

Bacterial counts. In an anaerobic chamber, serial 10-fold dilutions of the chemostat samples were prepared in PRAS diluent. Aliquots (0.1 mL) of the dilutions were plated on PRAS bacteroides bile esculin agar (BBE; Summanen *et al.*, 1993) to enumerate presumptive *B. fragilis* group members (BfG). It should be noted that by using BBE, the count of BfG underestimates the total population of bacteroides, since the most common component of this group, *B. vulgatus*, does not hydrolyze esculin and so does not form black colonies on BBE. Only black colonies were counted. Plates were incubated under anaerobic conditions for 2 to 3 days at 37°C. MacConkey and bile esculin azide agars (BEA; Difco, 1984), for the cultivation of presumptive *E. coli* and enterococci respectively, were inoculated and incubated under aerobic conditions at 37°C for 18 to 24 h. Plates with 30 to 300 colonies were counted. Counts were expressed as colony-forming units (CFU)/mL chemostat fluid.

Susceptibility to ciprofloxacin and trovafloxacin. BfG, *E. coli*, and enterococci were enumerated as follows. Plates with 30 to 300 colonies were replica plated onto BBE (BfG), MacConkey (*E. coli*), or BEA (enterococci) agar, with and without CI added to the agar, using sterile velvet pads and a replicating block and collar (Replicatech Inc., Princeton, NJ). The concentrations of CI added to the medium for each bacterium tested are specified under Results section. The last plate in each series to be inoculated was a no-drug control, employed to prove that when colonies failed to grow on CI (or trovafloxacin), it was not because of a failure of the replica plating. The BBE plates were incubated under anaerobic conditions for 2 to 3 days at 37°C. The antibiotic-supplemented MacConkey and BEA plates were incubated under aerobic conditions for 18 to 24 h at 37°C.

In addition, a series of BBE plates supplemented with trovafloxacin (2, 4, and 8 μg TV/mL; Pfizer, New York, NY) were inoculated with 30–300 colonies, also by replica plating. The plates were incubated under anaerobic conditions for 2 to 3 days at 37°C. This assay was run twice.

SCFA. The extraction procedure was based on that of Holdeman *et al.* (1977). Under a laminar flow hood, 1 mL of test sample was added to a screw-cap tube containing 0.4 g NaCl and 0.2 mL 50% H_2SO_4. Ether (1 mL) and internal standard (1% caprylic acid; 0.05 mL) were added to the tube. The mixture was mixed for 30 s, centrifuged at 1625*g* for 2 min, and then placed in a freezer at less than −70°C for a minimum of 10 min and maximum of 1 h. The ether layer was removed to sealed vials for analysis by gas chromatography. A HP 5890 series II gas chromatograph equipped with a flame ionization detector and a Stabilewax-DA column (30 m, 0.25 mm i.d., 0.25 μM df; Restek, Bellefonte, PA) was used. The carrier gas was helium (1 mL/min), the hydrogen flow rate was 32 mL/min, and the air flow rate was 380 mL/min. The temperature of the injector and detector ports was 250°C and the oven temperature began at 50°C and was raised 10°C/min up to 220°C for each sample run. The syringe was rinsed thoroughly between each analysis. Chromatograms were compared to a standard curve for each analyte in a volatile acid standard mix (Supelco, Bellefonte, PA). Concentrations were determined using a HP 3396B integrator. Total SCFA concentrations were expressed in molarity. Individual analytes were expressed as the percentage of the total, i.e., the percent molar ratio.

Statistical analysis. Four chemostats were run. Data obtained from samples taken from each chemostat during days 17–24 (i.e., after steady state was achieved, but before the exposure to CI) were pooled to create a "mean pretreatment" level for each parameter studied. The collected pretreatment values yield a regression line in time that, when in steady state, by definition, should have no slope. This was checked for every parameter assayed by comparing the slope of the observed regression line with a line with no slope, representing

steady state. For no parameter did these two differ significantly ($P \leq 0.05$) and so all parameters were shown to be at steady state prior to the introduction of CI.

Accordingly, the calculated mean pretreatment levels were plotted as horizontal lines extrapolated into the exposure period and bordered by a 95% prediction interval (PI_{95}). The prediction intervals were computed using both within chemostat and between chemostat variation. It was concluded that CI had no impact on the parameter being followed if data for the exposure periods fell outside the PI_{95} only 1 time in 20. If the incidence was greater than 1 in 20, it was concluded that CI had a measurable and significant impact on the steady-state level of that feature. When considering our results, we discounted the significance of single, isolated points lying outside the PI_{95}.

This approach assumed that all four pretreatment data sets were based on essentially identical steady-state floras.

RESULTS

Antibiotic Exposure

The effects of ciprofloxacin on bacteria in the chemostat system were related to the concentration of drug introduced into the system. We assumed that the bioavailability of the drug in each culture vessel, while not necessarily 100% of the mass added, was similar between culture vessels. No attempt was made to determine the drug concentration or bioavailability in the individual culture vessels, only in the medium as it passed from the reservoir to the culture vessel.

The mean bioassay test results for concentrations of drug in the medium reservoir are reported in Table 1, along with the amount of CI weighed into the chemostat. These results show that the amount of CI weighed into the chemostat medium and the level assayed from the medium were similar. Therefore, all the results of the microbiological endpoints are expressed in terms of the amount of test compound weighed into the medium and not on the levels determined by bioassay of the medium reservoirs. In addition, Brumfitt *et al.* (1984) found a mean of 0.891 mg CI/g feces in 12 volunteers given 1 g of CI daily for 1 week. Assuming that Brumfitt's ratio of oral intake to fecal concentration holds true at low levels of consumption, our test concentrations approximated human CI intakes of 0, 0.48, 4.8, and 48 mg/day.

TABLE 1
Levels of Ciprofloxacin Added to Medium and Measured by Bioassay

Ciprofloxacin level (μg/mL)	
Added	Assayed ± SD ($n = 3$ or 4)
None	Not found
0.43	0.77 ± 0.26
4.30	4.30 ± 0.52
43.00	47.64 ± 10.58

Bacterial Counts

BfG. Although the preexposure counts of BfG varied from $10^{4.4}$ to $10^{7.0}$ CFU/mL, the effect of CI on the BfG was apparent for at least the two higher concentrations (Fig. 1a). The decrease of BfG counts was dose dependent and significant at 4.3 and 43 μg/mL. At 0.43 μg/mL there was a 10-fold reduction in the BfG count within 2 or 3 days of CI exposure; however, this may have represented nothing more than the pretreatment variation of bacterial counts mentioned above. At 4.3 μg/mL, the count fell to 10^2 cfu/mL within 48 h of CI administration. Within 72 h of exposure to 43 μg/mL of CI, BfG counts were below detectable limits. This is shown in Fig. 1a as a count of 10^1 CFU/mL, the minimum level of detection.

Escherichia coli. Significant and dose-dependent effects of CI on *E. coli* counts were seen at all CI concentrations tested. Before adding CI to the medium, the *E. coli* counts ranged from $10^{5.3}$ to $10^{7.6}$ CFU/mL (Fig. 1b). Three days after adding 0.43 μg/mL, *E. coli* counts decreased approximately 100-fold. The same effect was observed after 48 h with 4.3 μg/mL, and eventually the count fell as low as 10^3 CFU/mL. Similarly, by the second day of exposure to 43 μg/mL, counts fell to 10^2cfu/mL.

Enterococci. Enterococci levels were $10^{4.1}$ to $10^{6.1}$ CFU/mL in all four chemostats (Fig. 1c). They were unaffected by any of the three CI medium concentrations.

Determination of Ciprofloxacin Resistance

Resistance as we use it is defined as the number of colonies growing on medium containing CI, expressed as the percentage of the number of colonies on the replica plate without CI.

BfG. The MIC_{90} of CI for BfG is 8 μg/mL (Schentag and Scully, 1999) and for other bacteroides is 4 μg/mL (Wolfson and Hooper, 1989). Consequently, we tested BfG resistance levels at 2, 4, and 8 μg/mL CI. Virtually no isolates (<1%) of BfG were resistant to 2 μg/mL before the exposure period (Fig. 2a). However, within 5 days of exposure, over 95% of BfG isolated from the 0.43 μg/mL chemostat were resistant to 4 μg/mL. During the same period, samples from the chemostats exposed to 4.3 or 43 μg/mL CI yielded too few or no isolates on plating, making susceptibility testing impossible.

Escherichia coli. The MIC_{90} of CI for *E. coli* is 0.01–0.25 μg/mL (Schentag and Scully, 1999). We assayed at 0.02, 0.04, 0.08, and 0.16 μg/mL. Possibly because of the lowered *E. coli* counts mentioned earlier, the resistance data were based on relatively few isolates. The results

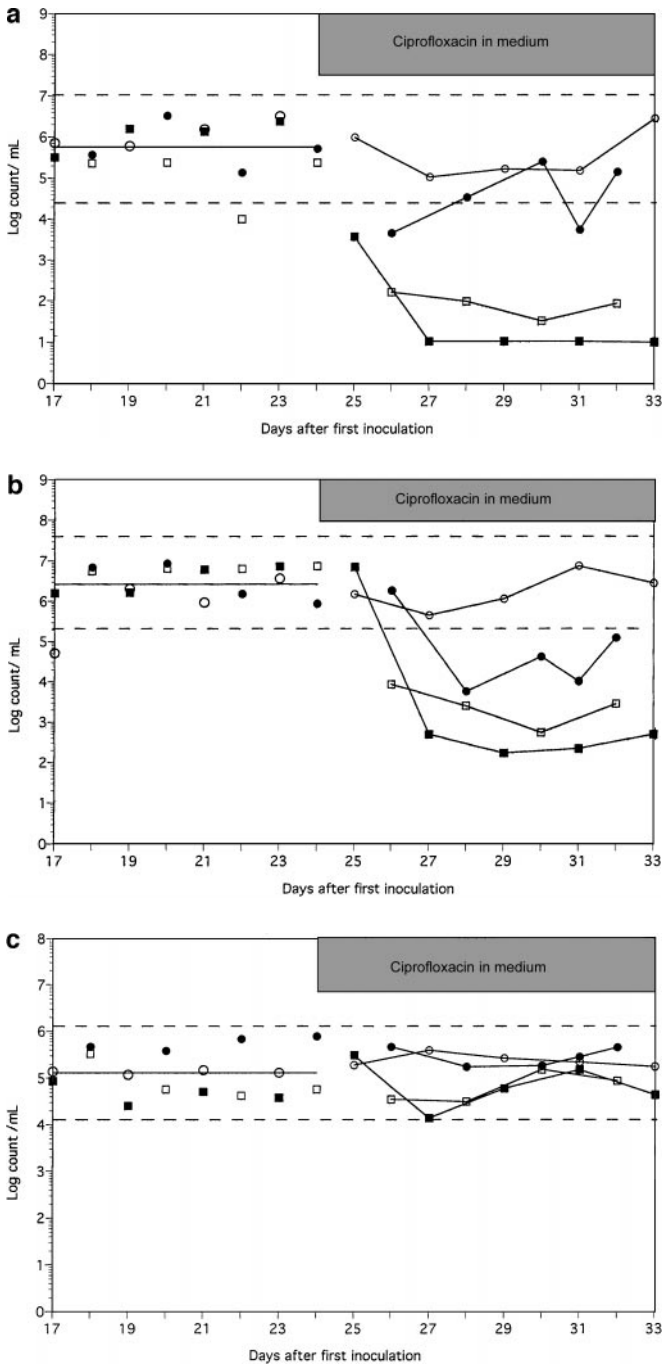

FIG. 1. Effect of ciprofloxacin on bacterial populations in a chemostat model of the human colonic flora. (a) *Bacteroides fragilis* group; (b) *Escherichia coli*; and (c) enterococci. —, pretreatment mean; - - -, 95% predicted interval. ○, no drug; ●, 0.43 μg/mL; □, 4.3 μg/mL; ■, 43 μg/mL.

fluctuated widely and followed no pattern (Fig. 2b). Interpretation was not possible.

Enterococci. The MIC_{90} for *Enterococcus faecium* is 2–8 μg/mL CI (Schentag and Scully, 1999). We assayed resistance at 2, 4, and 8 μg/mL. In the control (no drug) chemostat, the level of inherent resistance was low; less than 20% of isolates were resistant to 2 μg/mL. Resistance to higher levels of CI was not seen. No increase in resistance levels was seen in the chemostats treated with CI (Fig. 2c).

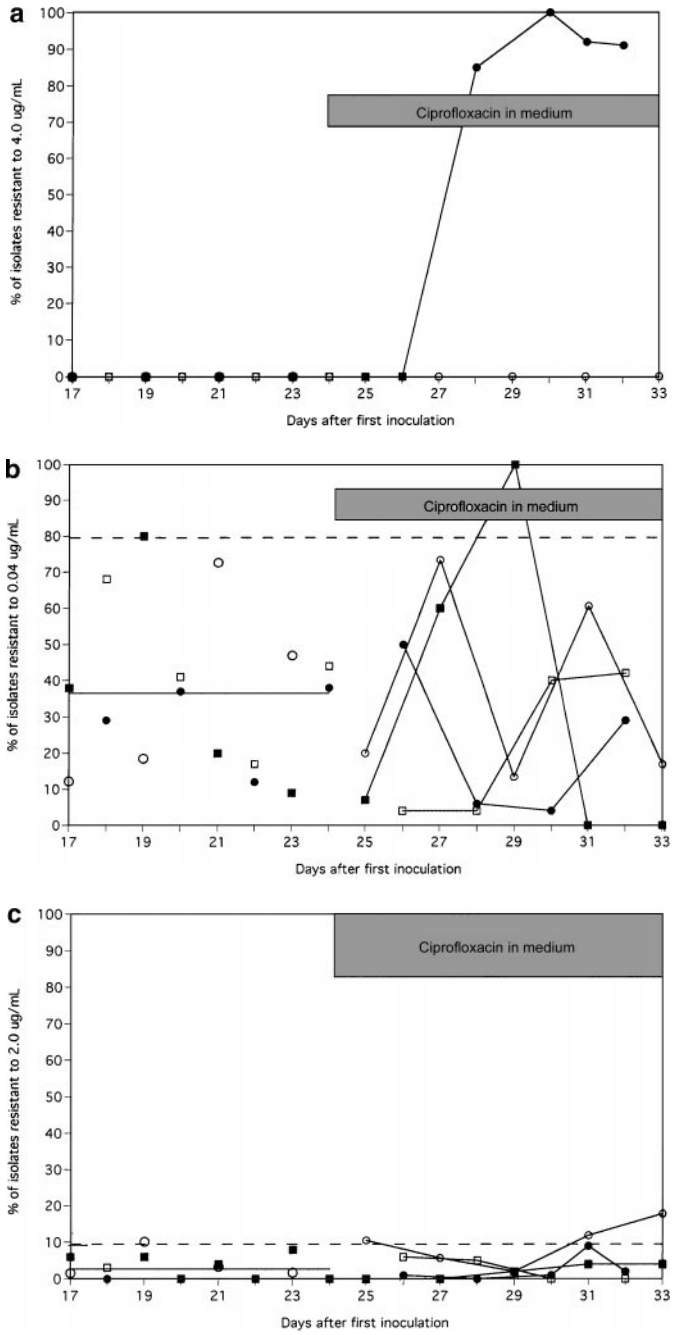

FIG. 2. Effect of ciprofloxacin on bacterial resistance to ciprofloxacin in a chemostat model of the human colonic flora. (a) *Bacteroides fragilis* group (4 μg/mL); (b) *Escherichia coli* (0.04 μg/mL); and (c) enterococci (2 μg/mL). —, pretreatment mean; - - -, 95% predicted interval. ○, no drug; ●, 0.43 μg/mL; □, 4.3 μg/mL; ■, 43 μg/mL.

Resistance to Trovafloxacin among the BfG

Trovafloxacin (TV), a relatively new naphtheradone FQ, was the first FQ with activity against anaerobes approved for use in the United States. Based on MIC_{90} data, TV has significantly greater activity than CI against anaerobes (Schentag and Scully, 1999). Because we showed that sub-MIC levels of CI (0.43 μg/mL) selected for >95% resistance among the BfG to 4 μg/mL, we also tested to determine if there was an increase in resistance to TV.

BfG were isolated from the chemostat before the dosing with 0.43 μg/mL of CI and 4 days after the start of the exposure period. Four further serial replica platings were done to test the stability of any resistance in the absence of CI. A fifth replica plating was done onto plates supplemented with TV or CI. BfG isolated before CI exposure were sensitive to both TV and CI. Conversely, when isolated during days 28–32 of the exposure period, 90–100% of the isolates were resistant to 4 μg/mL CI (Fig. 2a) and 2 μg/mL TV (data not shown). Furthermore, resistance to both CI and TV was stable in the absence of drug during four serial subcultures.

SCFA

There was no effect of CI on the combined total SCFA levels. These remained at approximately 48 mM regardless of the presence or absence of CI (Fig. 3). Of the individual components of SCFA, acetic and propionic acids, as a proportion of the total, remained unchanged (Figs. 4a and 4b). However, butyrate was significantly higher in the 43 and 4.3 μg/mL chemostats (Fig. 4c) and minor SCFAs were significantly lower at 43 μg/mL (Fig. 4d).

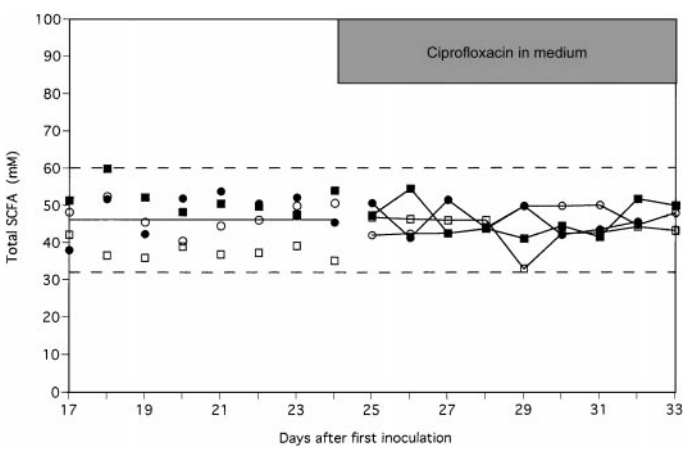

FIG. 3. Effect of ciprofloxacin on total short-chain fatty acids in a chemostat model of the human colonic flora. —, pretreatment mean; - - -, 95% predicted interval. ○, no drug; ●, 0.43 μg/mL; □, 4.3 μg/mL; ■, 43 μg/mL.

DISCUSSION

Bacterial Counts and Resistance

We tested CI exposure levels of 0, 0.43, 4.3, and 43 μg/mL in human microflora chemostat systems and estimated these to be equivalent to human intakes of 0, 0.48, 4.8, and 48 mg/day, respectively. In contrast, in 11 studies of patients and volunteers (Edlund and Nord, 1999; Terg *et al.*, 1999), the lowest daily dose of CI was 200 mg/day or more than fourfold our highest dose. (The highest dose in the clinical trials was 1500 mg/day.) Common to our study and all of the clinical trials was the finding that CI significantly reduces levels of *E. coli*. At chemostat exposure levels of 4.3 and 43 μg/mL CI, we observed between 1000 and 10,000-fold decreases in *E. coli* counts within 3 days of exposure. Even at 0.43 μg/mL, there was a clear though less significant reduction of approximately 100-fold.

Our finding of no observed CI effect on enterococci (Fig. 1c) matches that of 5 of 10 clinical studies referenced by Edlund and Nord in 1999, while in the other 5 clinical studies the observed reduction was less than 100-fold.

In addition to the effect on *E. coli*, we also saw a dose-dependent reduction of BfG levels (Fig. 1a) at 4.3 and 43 μg/mL. Although CI is primarily intended for the treatment of infections caused by aerobic and facultative anaerobic bacteria, this study showed that the incidence of both the most common aerobe (*E. coli*) and the most common anaerobes (BfG) was significantly reduced at lower than therapeutic levels of CI *in vitro*. This may correspond to the reduction in anaerobe counts seen in some clinical trials, including Brumfitt *et al.* (1984), albeit at much higher exposure levels than in our chemostat trial. The change in susceptibility of BfG to CI that we observed was stable over four subcultures in the absence of CI.

We saw no sign of decreased susceptibility to CI among *E. coli* as was reported in 2 of 10 clinical studies (Edlund and Nord, 1999; Terg *et al.*, 1999). Instead we found the percentage of *E. coli* isolates from the various chemostats to be highly variable, even before the exposure period began. Once started, the actual number of *E. coli* isolated was often too low for reliable determinations of susceptibility (Fig. 2b). Also, we saw no rise in enterococcal resistance to CI. It remained at less than 20% at all times (Fig. 2c).

Since the MIC_{90} of CI for *E. coli* is lower than 0.43 μg/mL, the observed reduction in *E. coli* counts should have been expected. However, the MIC_{90} of CI to enterococci is 2–8 μg/mL, which is equaled or exceeded in the 4.3 and 43 μg/mL chemostats. Therefore, levels of enterococci might also have been expected to fall. They did not and we have no ready explanation.

On the other hand, and in common with Brumfitt *et al.* (1984), we did see increased resistance among

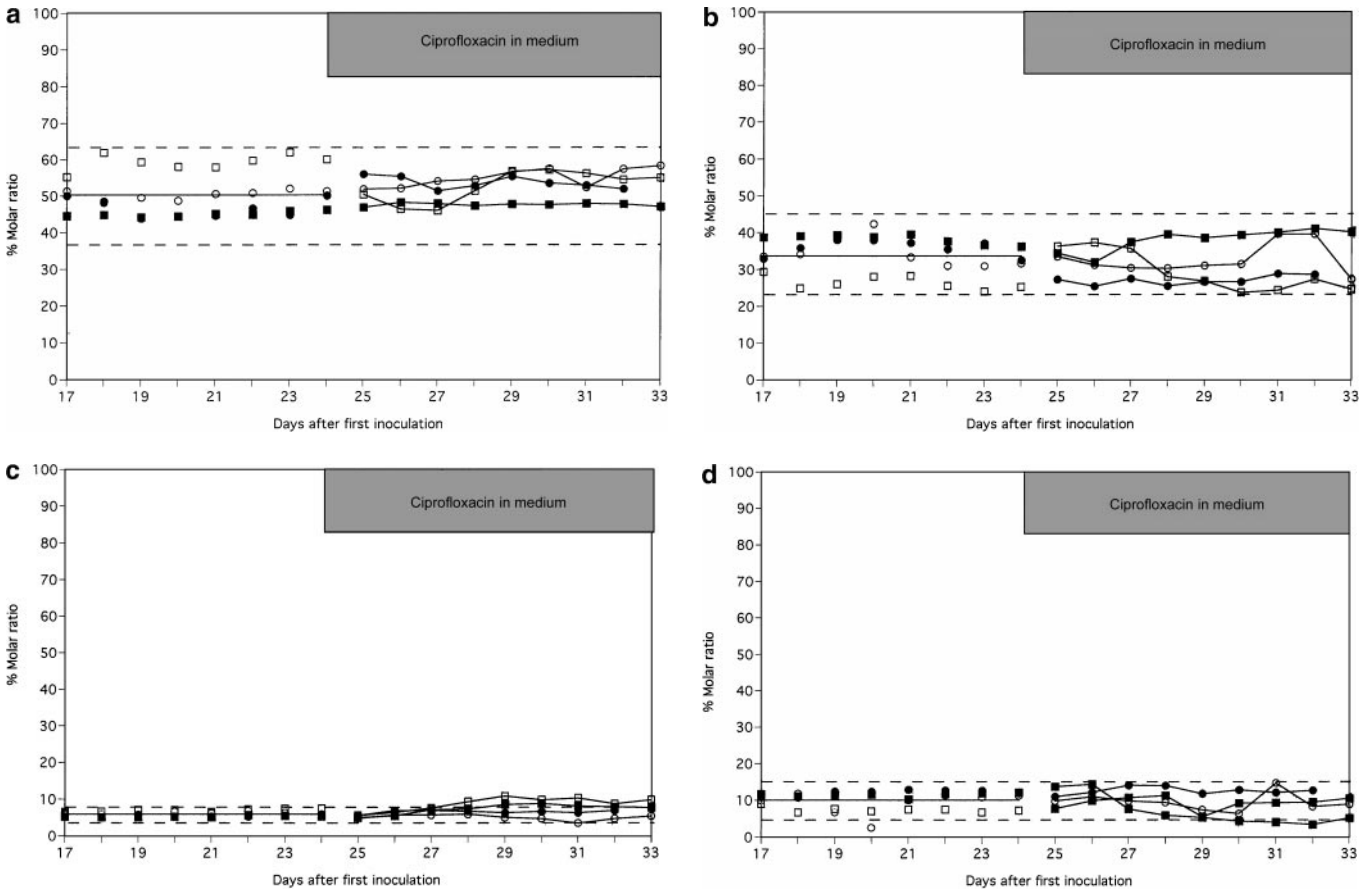

FIG. 4. Effect of ciprofloxacin on relative molar concentrations of short chain fatty acids in a chemostat model of the human clonic flora. (a) Acetate, (b) propionate, (c) butyrate; and (d) minor SCFA. —, pretreatment mean; - - -, 95% predicted interval. ○, no drug; ●, 0.43 μg/mL; □, 4.3 μg/mL; ■, 43 μg/mL.

anaerobes. BfG normally has an MIC_{90} of 8 μg CI/mL (Schentag and Scully, 1999; Wexler *et al.*, 1992). So, the effective elimination of BfG from the chemostat at 4.3 and 43 μg/mL CI should have been anticipated. More significantly, at 0.43 μg/mL CI (less than 10% of the MIC_{90}) the BfG counts decreased, but not always significantly. Furthermore, of the BfG isolated after exposure to 0.43 μg/mL, more than 90% were resistant to a nominal plating exposure of 4 μg CI/mL. This represents a fundamental change in the ecology of the most abundant component of the flora on exposure to a subtherapeutic dose. It may not, however, represent a potential clinical problem, as it is unlikely that BfG infections would be treated with CI.

Of greater concern, is the cross-induction of resistance to a newer FQ, TV, intended for the treatment of anaerobic infections. According to Schentag and Scully (1999), the MIC_{90} of TV for *B. fragilis* is between 0.25 and 2 μg/mL. All the isolates growing on 4 μg/mL CI were also able to grow on TV (2 μg/mL) and more than 50% grew on 4 μg/mL (data not shown).

It should be noted that resistance was assessed in this study by replica plating from plates containing between 30 and 300 colonies onto plates with the same medium containing different concentrations of CI. Resistance was expressed as a relative measure; i.e. the percentage of colonies growing on medium containing CI compared to the control plates without CI. This method captures changes in the populations of bacteria with respect to their susceptibility to various concentrations of CI in the medium. Since this method relates bacterial counts on the CI plates with counts on control plates, the choice of the plating medium used is of less importance than it would be for establishing minimum inhibitory concentrations.

SCFA

We saw no changes in combined total SCFA production (Fig. 3) or in the levels of acetate and propionate (Figs. 4a and 4b), the two most abundant SCFA. We did find, however, that butyrate was higher in the 43

and 4.3 μg/mL chemostats (Fig. 4c) and, though significant, the increase was only from about 7 up to about 10%. Conversely, minor SCFAs were slightly but significantly lower at 43 μg/mL (Fig. 4d). So, despite the large reductions in the levels of both BfG and *E. coli* associated with all levels of CI, this moderate elevation in butyrate levels at the expense of the minor SCFA is the only confirmation of any change in the bacterial population based on metabolic parameters. We are unaware of any published reports of studies into the effects of CI on SCFA.

The levels of combined total SCFA determined in this study ranged from 40 to 60 mM, which is much lower than other reported values (120–160 mM) in similar continuous culture systems (Hoverstad *et al.*, 1984; Cummings and Macfarlane, 1991). This difference in SCFA ranges could be due to differences in the media in the chemostats, the speeds with which the media were pumped through the chemostats, the fecal inocula, or some combination of these factors.

Model

Although the complexity of the chemostat model system restricts the number of replications in each treatment group, our study showed that the model is capable of detecting dose-related effects of residue levels of CI on microbiological endpoints in human intestinal flora *in vitro*. A higher number of replications would increase the statistical power of these types of studies.

The way CI was added to the culture vessels raises an issue. In the present study, each culture vessel changed from zero drug concentration to its final concentration over an equivalent period of time. Therefore, the period of exposure was constant, but the slope of the increase in drug concentration was different between culture vessels. This difference in slope could lead differences that, while not necessarily important to final colony counts, could be of importance to both the determinations of relative antibiotic susceptibility. It might be possible to determine if the rate of exposure to increases in drug concentrations is more, less, or equally important to the period of exposure at the final concentration and adjust the experimental conditions accordingly.

Finally, relative determinations of antimicrobial susceptibility and resistance presented in terms of drug exposure can be informative, as illustrated in this report. However, it would increase the value (as well as the complexity) of the experiment to incorporate control strains as internal bioavailability standards when plating culture vessel samples for susceptibility determinations. This additional step would provide the internal quality control necessary to make comparisons between the drug susceptibility and resistance results of different studies performed in the same laboratory or the same experiments conducted in different laboratories.

CONCLUSION

Reductions in *E. coli* and BfG counts after exposure *in vitro* to 0.43 μg/mL CI and increased resistance to CI and TV among the surviving BfG are potentially adverse biological effects. Residual levels of a FQ in the food supply could pose a potential threat to the ecology of the human gut flora, possibly jeopardizing its normal functions. From the clinical perspective, increased resistance due to low levels of CI in the food supply could jeopardize the continued efficacy of TV for the treatment of anaerobic infections. The results of this study showed that low levels of CI, even dose levels lower than the traditional threshold ADI of 1.5 mg/person/day, altered the ecology of the human intestinal microflora in the model *in vitro* system.

ACKNOWLEDGMENT

The United States Food and Drug Administrations Center for Veterinary Medicine funded this work.

REFERENCES

Brumfitt, W., Franklin, I., Grady, D., Hamilton-Miller, J. M. T., and Iliffe, A. (1984). Changes in the pharmacokenetics of ciprofloxacin and fecal flora during administration of a 7-day course to human volunteers. *Antimicrob. Agents Chemother.* **26,** 757–761.

Cummings, J. H., and Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.* **70,** 443–459.

Difco (1984). *Difco Manual*, 10th ed., pp. 129–131. Difco Laboratories, Detroit, MI.

Edlund, C., and Nord, C. (1999). Effects of quinolones on intestinal ecology. *Drugs* **50,** 65–70.

FDA (1996). *Microbiological Testing of Antimicrobial Drug Residues in Food*, Guidance Document No. 52. Center for Veterinary Medicine.

Gibson, G. R., Cummings, J. H., and Macfarlane, G. T. (1988). Use of three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. *Appl. Environ. Microbiol.* **54,** 2750–2755.

Guerin-Danan, C., Andrieux, C., and Szylit, O. (1999). Storage of intestinal bacteria in samples frozen with glycerol. *Microb. Ecol. Health Dis.* **11,** 180–182.

Holdeman, L. V., Cato, E. P., and Moore, W. E. C. (1977). *Anaerobe Lab Manual*, 4th ed., pp. 134–148 Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Holdeman, L. V., Good, I. J., and Moore, W. E. C. (1976). Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. Appl. Environ. Microbiol. **31,** 359–375.

Hoverstad, T., Fausa, O., Bjorneklett, A., and Bohmer, T. (1984). Short-chain fatty acids in the normal human feces. *Scand. J. Gastroenterol.* **19,** 375–381.

Moore, W. E. C., and Holdeman, L. V. (1974). Human fecal flora: The normal flora of 20 Japanese-Hawaiians. *Appl. Microbiol.* **27,** 961–979.

Schentag, J. J., and Scully, B. E. (1999) Quinolones. In *Antimicrobial Therapy and Vaccines* (V. L. Yu, T. C. Merigan, Jr., and S. L. Barriere, Eds), pp. 875–901. Williams and Wilkins, Baltimore, MD.

Summanen, P., Barton, E. J., Citron, D. M., Strong, C., Wexler, H. M., and Finegold, S. M. (1993). *Wadsworth Anaerobic Bacteriology Manual*, 5th ed., pp. 161–181. Star Publishing Company, Belmont, CA.

Terg, R., Llano, K., Cobas, S. M., Brotto, C., Barrios, A., Levi, D., Wasen, W., and Bartellini, M. A. (1999). Effects of oral ciprofloxacin on aerobic gram-negative fecal flora in patients with cirrhosis: Results of short- and long-term administration with daily and weekly dosages. *J. Hepatol.* **29,** 437–443.

Wexler, H. M., Molitoris, E., and Finegold, S. M. (1992). In vitro activity of three of the newer quinolones against anaerobic bacteria. *Antimicrob. Agents Chemother.* **36,** 239–243.

WHO (1995). Toxicological evaluation of certain veterinary drug residues in food: Enrofloxacin. In *WHO Food Additives Series 34*, pp. 61–84. World Health Organization, Geneva, Switzerland.

Wolfson, J. S., and Hooper, D. C. (1989). Fluoroquinolone antimicrobial agents. *Clin. Microbiol. Rev.* **2,** 378–424.