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Ciprofloxacin at low levels disrupts colonization resistance of human fecal microflora growing in chemostats

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Abstract

We studied the in vitro effects of a range of ciprofloxacin (CI) concentrations on the human intestinal flora's colonization resistance (CR) to *Salmonella kedougou* NCTC 12173. Four steady state microbial communities were established in chemostats using inocula from a single pool of human feces. Three chemostats were exposed to CI (0.1, 0.43 and 5 μg/mL, respectively); one served as a no-drug control. The CR of each community was tested by three successive daily challenges of 10^8 *S. kedougou*, each delivered in a 1 mL bolus. There was no colonization of the no-drug chemostat. Likewise, after exposure to only 0.1 μg/mL CI there was no loss of CR and *S. kedougou* did not colonize. Conversely, both the 0.43 and the 5 μg/mL-exposed floras suffered a loss of CR and these chemostats were colonized. *S. kedougou* overgrew faster and reached higher counts in the presence of 0.43 than it did in the presence of 5 μg/mL. One possible explanation is that CI had a dose-dependent effect on both the challenge strain and CR. Thus, at higher levels, even though CR was disrupted by CI, so too was the growth of the challenge strain. Since exposure to CI elicited a dose-dependent reduction in *Escherichia coli* counts [Reg. Pharmacol. Toxicol. 33 (2001) 276] our new data suggest that *E. coli* may contribute to the CR against salmonella. We further conclude that, even at fecal levels below those reached during therapy, CI may impact the human gut flora sufficiently to facilitate colonization by *S. kedougou*.

Keywords: Fluoroquinolone; Antimicrobial; Antibiotic; Residue; Nurmi effect; Barrier effect; Competitive exclusion; Intestinal; Feces; Chemostat

1. Introduction

Antibiotics may enter the human food supply as residues in food produced from treated animals and may have unintended effects on human flora, especially in the large bowel. There are three broad effects with potential public health consequences. They are: (1) shifts in bacterial counts and biochemistry, (2) changes in the incidence of antibiotic resistant bacteria, and (3) changes in the ability of the resident flora to prevent colonization by potential enteropathogens. Earlier we described the effects of therapeutic and sub-therapeutic doses of ciprofloxacin (CI) on the first two parameters (Carman and Woodburn, 2001). The third is called variously the Nurmi or barrier effect, competitive exclusion and, the name used in this publication, colonization resistance (CR).

Although CI is not approved for veterinary use within the US, it is a fluoroquinolone (FQ) and other FQs are used in veterinary medicine. Thus, CI is the 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, its effects on the

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human intestinal flora may be comparable to those of CI. In preference to relying on the in situ metabolism of enrofloxacin to generate active CI, we used CI as our test compound and at each level tested, the CI added was the equivalent to the product of a defined, precise, and reproducible conversion of enrofloxacin to CI.

We conducted our study using chemostat models of the human large bowel. Chemostats are culture systems in which sterile medium enters and exits at a fixed rate and physico-chemical variables, such as pH and medium components, do not change. After inoculation there is a period of adjustment that culminates in a steady state or stable flora, characterized by a consistent biochemistry and composition. Thereafter, on the variation of a single parameter (in our case CI levels) the effects over time on the whole ecosystem can be monitored.

In chemostats modeling the exposure of human colonic flora to CI, 0.43 μg/mL (a sub-therapeutic or "residue" level) produced a significant reduction in the number of bacteroides and *Escherichia coli*. Higher levels produced more pronounced but otherwise similar effects (Carman and Woodburn, 2001). This decrease in components of the resident flora may represent the "opportunity" that colonizers must exploit to become established in communities that might otherwise exclude them. Just what the opportunity is in terms of nutrition, habitat, etc. is not known. This report addresses the effect of exposure to CI on CR against *Salmonella kedougou* using virtually the identical chemostat model and a similar range of CI levels to those used by Carman and Woodburn (2001).

2. Materials and methods

2.1. Chemostat

The chemostat model has been thoroughly described before (Carman and Woodburn, 2001). The medium contained proteins, peptides, plant polysaccharides, bile acids, cholesterol, hemin, a reducing agent, and mineral salts. Culture fluid (500 mL) was stirred, sparged with nitrogen, and kept at 37 °C and between pH 6.4 and 6.6. Fresh medium was pumped into the culture vessel at 35 mL/h, a dilution rate of 0.07/h. Effluent left each chemostat via a side arm. Four chemostats were run simultaneously. To equilibrate them, they were run for 24 h before inoculation.

2.2. Inoculum

TechLab's Institutional Review Board approved collection of feces. Seven adult donors were recruited. None reported having received antibiotics within the preceding 12 weeks. Nor did any report having diarrhea or other intestinal tract problems during those weeks. All considered themselves to be in good health and consumers of non-vegetarian diets typical of North Americans. Each defecated into a sealable plastic bag and their sample was placed on ice in an anaerobic chamber. Within 120 min of defecation the samples were pooled, supplemented with glycerol (10% final concentration by weight: (Guerin-Danan et al., 1999), homogenized and dispensed into stoppered glass tubes. These tasks were done inside an anaerobic chamber so when the samples were frozen at $\leqslant -70$ °C they were still under an anaerobic atmosphere. To make inocula, feces were thawed at room temperature for 1 h. Inside the chamber they were suspended in 4 times their own weight of prereduced, anaerobically sterilized (PRAS) diluent (Holdeman et al., 1977). Chemostats were inoculated with 50 mL of the suspension containing 10 g feces on days 1, 3, and 5 of the study by injection via a septum in each vessel lid using syringes filled inside the chamber.

2.3. Ciprofloxacin levels

CI was provided by the Bayer Corporation (West Haven, CT). On day 25, CI dissolved in sterile medium was added to each of the medium reservoirs to achieve the nominal test levels, 0.1, 0.43, and 5 μg/mL. We chose these levels for several reasons. The minimum inhibitory concentration of CI towards *S. kedougou*, our challenge strain, was $\leqslant$0.125 μg/mL (see below for details), warranting the 0.1 μg/mL level. The 0.43 μg/mL dose is used to allow some direct comparison with our earlier findings and because it is known to severely reduce the population of *E. coli* in our model (Carman and Woodburn, 2001). Our earlier study into the effects of CI gave only limited insight into a high dose that was likely to disrupt CR yet still be helpful by being within range of the NOEL. We believed that a true therapeutic dose, though sure to be effective, was of limited value in titration. Instead we ran a 5 μg/mL dose. Supplemented medium was pumped into the chemostat vessels at 35 mL/h, thus approximately 48 h was required for the concentration of CI in each to reach its nominal test level. A no-drug control (0 μg/mL) was also run. CI was given for 7 days (i.e. until the end of day 31). The CI concentrations were assessed in triplicate by bioassay (Carman and Woodburn, 2001). Standard lawns of *E. coli* ATCC 11775 were grown on Wilkins Chalgren agar. The lawns were overlaid with discs wetted with known volumes of standard concentrations of CI or medium, with or without CI, that was removed during passage from the medium reservoirs to the chemostat culture vessels. Thus the sample contained all biologically active CI, whether bound or unbound to particulates. The minimum level of detection was 0.5 μg/mL.

Using HPLC it is possible to recover more than twice as much total CI from human feces as active CI recovered by bioassay (Brogard et al., 1985; Jehl et al.,

1987). In pigs (Wiuff et al., 2002) the bioassay recovered about half the total. The work of van Saene et al. (1988) strongly suggests that the shortfall is inactive CI bound to components of feces. Despite these findings, we used a bioassay to measure the CI in chemostats, arguing that when assessing an antimicrobial activity, it makes good sense to discuss concentrations in terms of active test compound not total recovery. So to fix test levels we referred to Brumfitt et al. (1984), who, using a bioassay, found a daily dose of 1 g to 12 healthy volunteers led to a mean CI fecal level of 0.891 mg/g. We used their bioassay data to extrapolate from oral doses of interest to a predicted test levels in the chemostat (Carman and Woodburn, 2001).

Most workers studying the antimicrobial effects of CI and other fluoroquinolones—not their pharmacokinetics—use a bioassay (Beneni and Minelli, 1995; Bergan et al., 1986; Brismar et al., 1990; Brumfitt et al., 1984; Carman and Woodburn, 2001; Collins et al., 1999; Esposito et al., 1987a,b; Garcia-Calvo et al., 2001; Goodman et al., 1986; Janin et al., 1987; Leigh et al., 1988; Liu et al., 2000; Ljungberg et al., 1990; Pecquet et al., 1986, 1987, 1990; Steinbakk et al., 1992). Some though use HPLC or both (Delsol et al., 2004; Krueger et al., 1997, 1999; McConville et al., 1995; Ritz et al., 1994).

2.4. Timetable

The chemostats were allowed to reach steady state and CI (0, 0.1, 0.43, and 5 μg/mL, respectively) was added to the medium from day 25 to 31. Each chemostat was challenged with 10^8 *S. kedougou* on the mornings of days 31 (after removal of samples for various assays), 32, and 33.

To harmonize this study with the earlier work (Carman and Woodburn, 2001) six samples (10 mL each) were collected daily from each culture vessel from day 17 through 32. Each aliquot was mixed with glycerol (Guerin-Danan et al., 1999) and flushed with anaerobic gasses for 5 min before freezing at $\leqslant -70\,^{\circ}C$.

2.5. MIC of ciprofloxacin towards Salmonella kedougou NCTC 12173

Using the standard agar dilution method proscribed by the NCCLS (1993) we twice measured the in vitro minimum inhibitory concentration of CI towards *S. kegdougou*. Both times the MIC was $\leqslant 0.125$ μg/mL. It should be noted that the NCCLS MIC method is intended only for clinical isolates. The NCCLS methods may exaggerate the susceptibility of *S. kedougou* to an antibiotic such as CI, that works at the DNA level, by having the test strain growing alone, starting from a low-density inoculum of actively dividing cells plated onto rich medium, as opposed to growth in the chemostat, at a slow rate, in nutritionally poor medium and in a very aggressive competition with other fecal microbes.

2.6. Challenge with Salmonella kedougou NCTC 12173

The poultry industry, long plagued by the public health and financial consequences of salmonella colonization of newly hatched chicks, has developed an international standard for the testing the efficacy of protective floras (Mead et al., 1989). Whether or not a flora confers colonization resistance to newly hatched birds is tested by oral challenge with the non-host specific *S. kedougou*, an uncommon serotype occasionally associated with food poisoning. The approach was derived from procedures to measure colonization resistance in poultry (Mead et al., 1989) that were later adapted for use in chemostat trials (Nuotio and Mead, 1993). We confirmed that *S. kedougou* could grow in

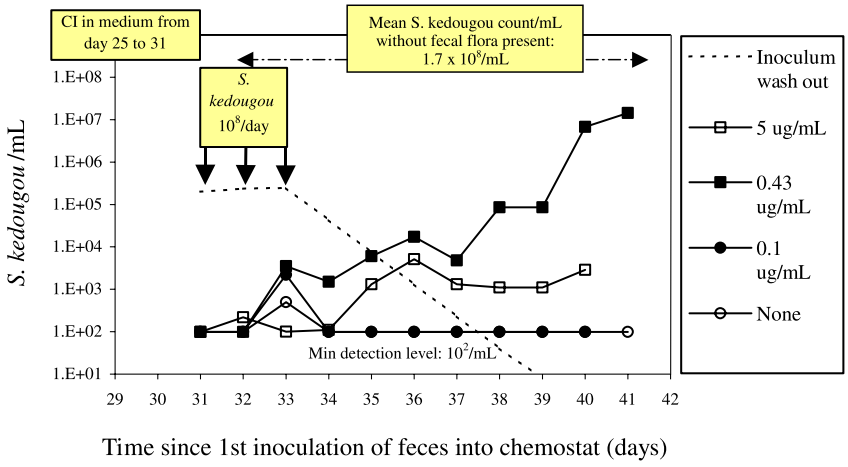

Fig. 1. Effect of ciprofloxacin (CI) on colonization resistance to *S. kedougou* in a chemostat model of the human colon. The dashed line (-·-·-·-·-) shows the level (1.7×10^8/mL) reached after the three additions of *S. kedougou*, each of 10^8 cells, and in the absence of any fecal inoculum. The dotted line (-----) represents the theoretical count that would have resulted from the *S. kedougou* inoculations. See Table 1 for details of the nominal and assayed levels of CI in the medium.

an otherwise uninoculated chemostat run under the conditions described above (Fig. 1). On two occasions we also determined the in vitro minimum inhibitory concentration (MIC) of ciprofloxacin for *S. kedougou* using a standard agar dilution method (NCCLS, 1993). The MIC was $\leqslant$0.125 μg/mL implying that growth in any of our chemostats exposed to CI would be the result of a loss of CR by that chemostat flora rather than due simply to a selective pressure exerted by CI and to which *S. kedougou* was inherently resistant.

To prepare the challenge material, *S. kedougou* was grown by batch culture and chemostat medium for 8 h at 37 °C. Aliquots were frozen at <−70 °C. After thawing three aliquots at room temperature, serial 10-fold dilutions of each were plated onto Brilliant Green Agar (Difco) supplemented with nalidixic acid (20 μg/mL; Sigma Chemical Co.). The mean count was $10^{8 \pm 0.12}$/mL. Each chemostat received 1 mL/d (i.e. 10^8 cells/d) on days 31, 32, and 33.

2.7. Salmonella counts

Inside the 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 Brilliant Green Agar supplemented with nalidixic acid (20 μg/mL; Sigma Chemical Co.). Following aerobic incubation at 37 °C for 18–24 h, cherry-red colonies, typical of salmonella, were counted (Mead et al., 1989). Whenever possible, plates with 30–300 colonies were counted. Counts were expressed as cells/mL.

3. Results

3.1. Antibiotic exposure levels

The amounts of CI added to each chemostat and the levels determined by bioassay are shown in Table 1. For comparison, Table 1 also gives the "equivalent daily intake" of CI required to achieve each of these levels in feces. These were calculated by extrapolation from the oral dose and fecal levels reported by Brumfitt et al. (1984). The nominal 0.1 and 0.43 μg/mL levels used in the chemostats were below the minimum level of detection (0.5 μg/mL). The mean observed CI level in the nominally 5 μg/mL chemostat was almost twice the anticipated level. So to provide some degree of consistency while discussing the data, we will characterize the CI levels in terms of the amount added rather than the amount recovered. No attempt was made to determine concentration or availability in the individual culture vessels, only in the medium as it passed from the reservoir to the culture vessel.

Table 1
Levels of ciprofloxacin added to medium and measured by bioassay

Ciprofloxacin level		
Nominal level added to chemostat medium (μg/mL)	Assayed ± SD (μg/mL; $n = 8$)	Equivalent daily intake (mg/person/d)
None	Not applicable	None
0.1	<0.5	0.1
0.43	<0.5	0.5
5.0	9.0 ± 1.1	6

3.2. Colonization with Salmonella kedougou

Fig. 1 shows the results of attempts to colonize the four chemostats with *S. kedougou*. The dashed line shows the *S. kedougou* levels reached after the three additions of *S. kedougou*, each of 10^8 cells, and in the absence of any fecal inoculum. The dotted line represents the theoretical count that would have resulted from the *S. kedougou* inoculations. It reflects a 0.07/h dilution rate and assumes that the cells neither died nor divided. For colonization to occur the rate of cell division must exceed the combined losses from death and wash out. Under these conditions, *S. kedougou* levels will exceed the theoretical count and will establish itself at a stable level. This occurs more quickly when the difference between losses and division are greater, though speedy progress to a steady state does not necessarily predicate a high count once at steady state.

During the challenge phase *S. kedougou* was recovered from all four chemostats. However, counts quickly fell in each, in three cases to below the minimum level of detection (<10^2/mL). It remained below the level of detection in both the no-drug control and the 0.1 μg/mL chemostats. Conversely, in the 0.43 and 5 μg/mL chemostats, the counts increased, only slowly at the higher dose (10^3/mL by day 40) but more quickly at 0.43 μg/mL (10^7/mL by day 41). By day 41, any residuum of inoculum should have been washed out 60 h earlier. In the 0.43 μg/mL chemostat the counts eventually reached as high as 10^7/mL. For reference, in a CI-free chemostat inoculated with *S. kedougou* alone the mean count at steady state was $10^{8.1}$/mL.

4. Discussion

S. kedougou grew well in an otherwise uninoculated chemostat and reached levels of 10^8/mL after 48–72 h. On the other hand, it did not grow in the no-drug control chemostat showing that the climax community in a chemostat inoculated with feces but receiving no CI exerted effective colonization resistance. At 0.1 μg/mL, a very low level, CI did not demonstrably affect CR and *S. kedougou* did not colonize at that level. Conversely, at 0.43 and 5 μg/mL CR was so reduced that *S. kedougou* colonized both chemostats. Paradoxically, colonization was more rapid at 0.43 μg/mL than at the higher level

(5 μg/mL). One explanation is that after CI was discontinued, it took longer to wash out from the 5 μg/mL chemostat than it did from the 0.43 μg/mL vessel. Taking into account the 35 mL/h flow rate and the initial concentrations in the sterile medium, the in vitro MIC of CI (0.125 μg/mL) was not reached in the 5 g/mL chemostat until 50 h after the drug was withdrawn or, put another way, not until 2 hours after the third and final challenge with *S. kedougou*. The residual level of CI in the 0.43 μg/mL would have dropped below the in vitro MIC 33 h earlier, i.e. 7 h before the second challenge with *S. kedougou*. It is possible that given sufficient time the counts in 5 mg/mL would have reached the same level as in the 0.43 μg/mL chemostat. With regard to CR, the "no effect level" for CI in this study was 0.1 μg/mL. By extrapolation from the relationship between oral dose and fecal level reported by Brumfitt et al. (1984), a NOEL of 0.1 μg/mL may be the equivalent of 0.1 mg/person/d, a level comparable with the ADI of 0.1 g/person/d (FDA, 2002) for enrofloxacin, a related FQ drug.

In our earlier chemostat studies (Carman and Woodburn, 2001) both the bacteroides and *E. coli* counts were reduced, sometimes below the level of detection, by exposure of the climax community to CI at a concentration as low as 0.43 μg/mL. Whether their elimination was due to killing of the target organisms or whether, at sub-therapeutic levels, the bacteria divided more slowly than in the absence of CI so that ultimately they were washed out is unclear. It matters little since both killing and wash out are potentially adverse ecological consequences of exposure to CI and both are possible in vivo responses in the human gut, a culture vessel that is partially emptied and partially refilled daily. Furthermore, because the responses we saw in the previous study were not confined to a single bacterial group and because we have only monitored a limited cross section of the flora, we cannot definitively identify that component of the flora responsible for CR. Nonetheless, given the similarities between *E. coli* and *S. kedougou*, it is tempting to credit CR against pathogenic *Enterobacteriaceae* on the non-pathogenic members of the family. Thus we suspect resident strains of *E. coli* in our model contributed to CR against *S. kedougou*.

Therapeutic levels of oral CI, as our interpretation requires, dramatically reduced the incidence of coliforms in the feces of healthy adults (Brumfitt et al., 1984). This result has been confirmed since many times since for both oral and intravenous CI (Bergan et al., 1986; Enzensberger et al., 1985; Holt et al., 1986; Krueger et al., 1997, 1999; Ljungberg et al., 1990; Maijer-Severs et al., 1990; Pecquet et al., 1990; Tillonen et al., 1999; van de Leur et al., 1997; van Saene et al., 1988), in healthy infants (Ljungberg et al., 1990) and in patients (Borzio et al., 1997; Brismar et al., 1990; Esposito et al., 1987a,b; Rozenberg-Arska et al., 1985; Terg et al., 1998). The response has been reproduced in healthy rats (Beneni and Minelli, 1995; Nosova et al., 1999; Peltonen and Eerola, 1992), in human flora associated mice (Gismondo et al., 1995) and in chemostats (Bernhardt et al., 1998; Carman and Woodburn, 2001). The response lasts only so long as CI is administered (Bergan et al., 1986; Brismar et al., 1990; Brumfitt et al., 1984; Esposito et al., 1987a,b; Gismondo et al., 1995; Krueger et al., 1997; Pecquet et al., 1990).

An increased incidence of resistance to CI in *E. coli* has been seen by some (Borzio et al., 1997; Carman and Woodburn, 2001; Terg et al., 1998; Wistrom et al., 1992) but not in all studies (Bergan et al., 1986; Gismondo et al., 1995; Pecquet et al., 1990; van de Leur et al., 1997). Had there been already resistant *E. coli* strains in our fecal inoculum, we might have expected to see overgrowth of these strains in the presence of CI and a parallel retention of colonization resistance to less resistant *S. kedougou*. In fact, CI at 0.43 μg/mL led to a more than 100-fold drop in levels of *E. coli* (Carman and Woodburn, 2001) and no increase in CR (Fig. 1). Thus, it appears our inoculum lacked any inherent resistance to CI. In this we were fortunate and in future would prescreen donors for resistance among target bacteria.

Most workers saw no effects of CI on enterococci levels (Beneni and Minelli, 1995; Carman and Woodburn, 2001; Enzensberger et al., 1985; Krueger et al., 1997), though on occasions elevated (Borzio et al., 1997) and reduced (Bergan et al., 1986; Brismar et al., 1990; Brumfitt et al., 1984; van Saene et al., 1988) counts have both been reported.

In general there are few apparent effects of CI on anaerobes (Beneni and Minelli, 1995; Bergan et al., 1986; Brumfitt et al., 1984; Enzensberger et al., 1985; Esposito et al., 1987a,b; Holt et al., 1986; Ljungberg et al., 1990; Rozenberg-Arska et al., 1985). A few researchers saw a drop in the bacteroides counts (Brismar et al., 1990; Carman and Woodburn, 2001; Maijer-Severs et al., 1990; Wistrom et al., 1992). Emerging resistance among bacteroides has been seen (Brumfitt et al., 1984; Carman and Woodburn, 2001). It is not always clear if the resistant strains that occur after treatment are native strains that have newly acquired resistance, or native strains that were resistant but not very common, or colonization by already resistant foreign strains. Unless the model is a closed system (e.g. a chemostat or a mouse inside an isolator) all three could occur, possibly in combination.

Whereas there is a consensus on the principal effects of CI on the gut flora when the drug is given at therapeutic level of 250 mg/d and higher, the effects of low doses are not often studied. For each model (human, rodent, human flora associated rodent, and chemostat) we can consider the lowest reported test dose and the effects it elicited. van de Leur et al. (1997) gave healthy volunteers 20 mg/d or for a 60 kg person, about 0.33 mg/kg/d. It led to the elimination of *E. coli* in 4 of 5 individuals. Germ free mice, seeded with human fecal flora, were

given 30 mg/kg/d (Gismondo et al., 1995). Not surprisingly for such a relatively high dose, there was the typical effect on *E. coli*, but nothing worthy of comment so far as an effect on anaerobes was seen. Rats given CI in their water (8 mg/100 mL) were estimated to consume about 9 mg/kg/d (Peltonen and Eerola, 1992). Their *E. coli* counts fell during the exposure period but returned to normal on withdrawal of CI. Carman and Woodburn (2001) saw an effect on *E. coli* at 0.43 μg/mL, which is the equivalent of about 0.5 mg/person/d or slightly less than 0.1 μg/kg/d. We are here reporting a loss of CR towards a member of the *Enterobacteriaceae* at the same dose, 0.43 μg/mL (0.5 mg/person/d or slightly less than 0.1 μg/kg/d).

Little work has been done on the activity of CI on host colonization resistance. There is no reason however to assume that CR against colonizers other *E. coli* is conferred by one and the same mechanism. In truth, it is likely that CR to each nominated bacterium will reflect the ecology of that bacterium. Consider antibiotic associated diarrhea (AAD) caused by toxin producing *Clostridium difficile*, a well-defined example of the loss of CR. In health, the mucin-degrading flora—entirely anaerobic—consumes mucin sugar residues that would otherwise be free to support the growth of *C. difficile* (Wilson and Perini, 1988). No such limiting nutritional requirements have been established for *E. coli* and thus it is unlikely CR towards *E coli* shares a mechanism with CR towards *C. difficile*. It is intriguing therefore that despite there being no AAD during trials in which CI was given to healthy volunteers (Bergan et al., 1986; Enzensberger et al., 1985; Maijer-Severs et al., 1990; Pecquet et al., 1990), fluoroquinolones, CI among them, have recently emerged as a class of antibiotics whose use significantly increases a recipient's risk of developing *C. difficile*-AAD (Crabtree et al., 1999; Yip et al., 2001). In fact, they have more than 4 times the risk associated with clindamycin, a drug notorious for causing AAD, and more than 8 times the risk for all other antibiotics (McCusker et al., 2003). In particular, extended exposure to CI is significantly associated with increased risk of *C. difficile*-antibiotic associated diarrhea (Lai et al., 1997), a serious and sometimes fatal nosocomial disease. One explanation may be that non-biliary excretion, through the mucosa into the bowel, leads to significant tissue concentrations of CI (Ramon et al., 1994, 1996). For example Brismar et al. (1990) found 38 μg/g in human colorectal mucosa, a high level possibly inhibitory to members of the normal flora intimately linked by mucin degradation to the intestinal mucosa.

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