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Running Title: Intestinal Impact of Fluoroquinolones

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ABSTRACT

Antibiotic treatment of lung infections may lead to the emergence of resistance in the gut flora. Appropriate dosing regimens could mitigate this adverse effect. In gnotobiotic rats harbouring intestinal *E. coli* and *E. faecium* populations, a lung infection by *K. pneumoniae* was instigated with two different sizes of inoculum to represent an early or a late initiation of antibiotic treatment. The rats were treated with marbofloxacin, a third generation fluoroquinolone by a single shot administration or a fractionated regimen over 4 days. Intestinal bacterial populations were monitored during and after treatment. At the infection site bacterial cure without any selection of resistance was observed. Whatever the dosage regimen, fluoroquinolone treatment had a transient negative impact on the gut *E. coli* population but not on that of *E. faecium*. The intestinal flora was colonized by the pathogenic lung bacteria and there was the emergence of intestinal resistant *K. pneumoniae*, occurring more often in animals treated with a single marbofloxacin dose than with the fractionated dose. Bacterial cure without resistance selection at the infection site with fluoroquinolone treatment can be linked to colonization of the digestive tract by the targeted pulmonary bacteria, followed by the emergence of resistance.

Key words.

Fluoroquinolones, Resistance, Intestinal flora, Dosage Regimen, Colonization.

INTRODUCTION

The emergence of antimicrobial resistance during antibiotic treatment can occur either in the infected organ system and/or in the endogenous normal gut flora (3). Antimicrobial agents including fluoroquinolones can be extensively excreted into the intestinal tract exposing the normal host flora to antimicrobial selective pressure (i.e. inhibition of competing microflora). This may lead to a secondary development of antibiotic-resistant gut organisms (2, 3, 17). *K. pneumoniae* is an important opportunistic pathogen implicated in nosocomial bacterial infections (19). Epidemiological studies have shown that the majority of *K. pneumoniae* infections are often preceded by colonization of the patient's gastrointestinal tract by the bacteria (9, 10). Recent reports suggest that fluoroquinolone-resistant *Klebsiella pneumoniae* isolates are common in many long-term care facilities and hospitals and are often associated with multidrug-resistant phenotypes (11, 22). The origins of this resistant *K. pneumoniae* gut subpopulation should deserve attention. A possible factor contributing to the emergence of a resistant subpopulation of *K. pneumoniae* in the gut could be an inadequate treatment of a prior *K. pneumoniae* infection with a secondary gut colonization by *K. pneumoniae*. This *K. pneumoniae* strain may then expand in the gut flora due to the selective pressure of the antibiotic reaching the gut lumen. Factors such as the concentration of the antimicrobial in the intestinal tract, the duration of the antimicrobial therapy, and the associated degree of disruption of the microflora may influence the likelihood that *K. pneumoniae* resistant strains will or will not emerge at the gut level (3, 21). In previous studies on rodent models of *E. coli* thigh infection and *K. pneumoniae* lung infection, we showed that the bacterial load at the start of antimicrobial treatment plays an important role in the enrichment of resistant strains at the infection site (4, 8). With a low inoculum, starting antimicrobial treatment early with marbofloxacin, a third generation fluoroquinolone extensively used in veterinary medicine, prevented mutant enrichment at the infection site whereas a late start of the antimicrobial treatment on a large inoculum, led to the enrichment of the resistant mutant subpopulation. Moreover, we showed that the

emergence of resistance was dependant on the total marbofloxacin dose and dosage regimen.

The aim of the present study was to assess, in a *K. pneumoniae* experimental infection model, the impact of different marbofloxacin dosage regimens on the commensal intestinal flora and to test the hypothesis that the critical site of emergence of resistance of a targeted lung pathogen during antibiotic treatment may be not the lung itself but the gut flora. With this as our aim, we developed a model of lung infection in gnotobiotic rats with two inoculum sizes of *K. pneumoniae* each treated with two different marbofloxacin dosage regimens. We chose to work with a gnotobiotic model harbouring a gram positive and a gram negative bacterial population in the intestine.

MATERIALS AND METHODS.

Bacterial strains and antibiotics.

The *Escherichia coli* and *Enterococcus faecium* strains used for the establishment of dixenic gut flora in rats came from pig samples from French slaughterhouses (AFSSA, Fougères) and *K. pneumoniae* ATCC 43 816 was used for the establishment of the lung infection.

Marbofloxacin powder, was kindly provided by Vetoquinol, Lure, France.

Klebsiella pneumoniae lung infection.

Germ-free male OFA rats (Charles River, L'arbresle, France), weight 170-200g, were housed individually in different sterile isolators, with a 12-h-light/12-h-dark cycle. Rats were fed ad libitum with an irradiated rodent chow (R03 40, UAR, Villemoisson, France) and were supplied with sterilized distilled water.

The germ-free status of the rats was checked immediately after their reception and during the period of acclimatization (see bacteriological procedures). After about one week, the rats were inoculated intragastrically with 1 mL of a saline (0.9% NaCl) suspension of *E. coli* (10^9 CFU/mL) strain and 1 mL of a saline (0.9% NaCl) suspension of *E. faecium* (10^9 CFU/mL).

The experimental lung infection was produced as previously described (1, 8). Briefly, the trachea was cannulated and the lungs were inoculated with 0.05 mL of a saline suspension (0.9% NaCl) of *K. pneumoniae* containing 2×10^6 CFU/mL (10^5 CFU total, Group A) or 2×10^9 CFU/mL (10^8 CFU total, Group B).

All animal procedures were conducted in accordance with accepted standards of animal care under the agreement number A 31909 for animal experimentation from the French Ministry of Agriculture.

Antimicrobial treatment.

Subcutaneous marbofloxacin treatment (MarbocylND, Vetoquinol, Lure, France) was started 4 hours (Group A) or 24 hours (Group B) after the lung infection. There were two modalities of

111 treatment: doses were administered either in one single administration (Groups A1 and B1,
112 n = 10 and n = 7 respectively) or the same total dose was fractionated into 4 daily
113 administrations over 4 days (Groups A2 and B2, n = 8 and n = 7 respectively). The total
114 marbofloxacin dose was 16 mg/kg, for group A and 64 mg/kg for group B. Stool samples
115 were collected at day 0, 4 and 7 after the first marbofloxacin administration for bacterial
116 analyses. The animals were sacrificed 7 days after the first marbofloxacin administration by
117 an intraperitoneal injection of sodium pentobarbital (DolethalND, Vetoquinol, France). The
118 lungs were aseptically removed and homogenized in 10 mL of 0.9% NaCl before
119 bacteriological analysis.
120
121 **Bacteriological procedures.**
122 **(*i*) MIC determination.** MICs were determined in triplicate for the bacteria by a broth micro
123 dilution method according to CLSI references methods.
124
125 **(*ii*) Faecal bacteriology.** Stool samples were diluted tenfold in distilled water and
126 homogenized. The germ-free status was verified on Schaedler agar supplemented with
127 sheep blood, Brain Heart Infusion (BHI) agar supplemented with sheep blood; and Malt
128 Extract Agar. During the study, 100 µL of faecal homogenates collected at days 0, 4 and 7
129 after the start of marbofloxacin treatment were plated on Slanetz and Bartley Medium for the
130 *E. faecium* strain, on Mac Conkey Agar for the *E. coli* strain, and on Mac Conkey Agar
131 supplemented with 0.3 µg/mL of marbofloxacin for resistant *E. coli*. and colonies were
132 counted after 24 hours incubation at 37°C for *E. coli* and 48 hours for *E. faecium*. The lowest
133 level of detection was 100 CFU/g faeces.
134
135 **(*iii*) Lung bacteriology.** Lung homogenates were plated on drug-free Mueller Hinton agar
136 plates containing 10% activated charcoal and 10% $MgSO_4$ to enumerate total bacterial
137 counts of *K. pneumoniae* and on Mac Conkey agar supplemented with 0.3 µg/mL
138 marbofloxacin to enumerate bacterial counts of resistant *K. pneumoniae*. Colonies were

139 counted after overnight incubation at 37°C. The lowest level of detection was 100 CFU/lung
140 and bacteria were considered eradicated below this level.

141 **Pharmacokinetics.**

142 Two satellite groups (Group C and D) of conventional male OFA rats (Charles River,
143 L'arbresle, France), weight 250-270g, were inoculated with 0.05 mL of an inoculum of
144 2×10^6 CFU/mL (Group C) or 2×10^{10} CFU/mL (Group D) of *K. pneumoniae*. Four hours
145 after the inoculation, group C was given 4 or 16 mg/kg of marbofloxacin by subcutaneous
146 administration. For the group D, subcutaneous marbofloxacin administration was 24 hours
147 after the *K. pneumoniae* inoculation and was 4, 16 or 100 mg/kg. The total amount of
148 excreted faeces was collected over 48 hours. Two to four rats were included per treatment
149 group. Stool samples were stored at -20°C until assayed for marbofloxacin by a high
150 performance liquid chromatography method with fluorescence detection (λ_{exc} = 295 nm, λ_{em} =
151 500 nm) (Agilent 1100) adapted from Schneider et al. (18). Briefly, for each sample time,
152 faeces were pooled, mixed with 2.5% trichloroacetic acid and centrifuged. The supernatant
153 was added to 1 mL of dichloromethane and mixed for 10 seconds. 0.2 mL of a mixture of
154 MeOH (2%HCl)/H_2O (90:10) was added to the organic layer and 100 µL of the supernatant
155 were injected into a C18e (Lichrospher, Merck, 5 µm 125x4 mm) column and eluted with a
156 phosphoric acid (0.01M)-triethylamine(0.004M) (pH = 2)/Acetonitrile gradient. The calibration
157 curve of marbofloxacin was established over the concentration range from 550 to
158 5000 ng/mL with a linear regression model. The accuracy varied from 94.8 to 113.87% and
159 the intra-day and inter-day precision was lower than 10.51% and 7.7% respectively. The limit
160 of quantification was 550 ng/mL. The samples were diluted to ensure that the concentrations
161 fell within the range of the calibration curve.

RESULTS AND DISCUSSION

Susceptibility studies.

The MIC of marbofloxacin was 0.032 µg/mL for both *K. pneumoniae* and *E. coli* and 2 µg/mL for *E. faecium*.

Faecal Pharmacokinetics.

Bacterial infections have been shown to alter the pharmacokinetics of drugs (5), including fluoroquinolones (7, 13). For this reason, we evaluated the amount of marbofloxacin excreted in the faeces in our model of *K. pneumoniae* lung infection. We observed that the amount of marbofloxacin excreted in the faeces of rats was proportional to the dose within each animal group (A or B), but differed between groups: the percentage of the marbofloxacin dose excreted in faeces was 4-fold less for the animals infected with the large *K. pneumoniae* inoculum than for those infected with the low inoculum (mean = 5% vs. 23% respectively, see Table 1). Consequently, taking into account the fact that groups B received a four times higher marbofloxacin dose than groups A (64 vs. 16 mg/kg), the amount of marbofloxacin excreted in the faeces was approximately the same in the two groups.

Effect of different marbofloxacin dosage regimens on survival rate and on the emergence of resistant *K. pneumoniae* in the lungs.

The amount of *K. pneumoniae* in each rat's lung and the percentage of animals with resistant *K. pneumoniae* at the end of the experiment for the two initial inoculum sizes and the different dosing regimens are shown in Table 2. All the rats infected with the low *K. pneumoniae* inoculum survived (groups A), and seven days after the start of the antimicrobial treatment, bacteria were not detected in the lungs of all the rats (except two) whatever the dosing regimen. For the rats infected with the high *K. pneumoniae* inoculum (groups B), the survival rate differed slightly according to the dosage regimen and was higher for the animals treated with the fractionated marbofloxacin regimen (group B2) than for animals treated with the one-shot dose (group B1). Nevertheless, the total bacterial population in the surviving

190 animals seven days after the start of antimicrobial treatment was almost the same for the two
191 dosing regimens.
192 More importantly, whatever the dosage regimen, there were no *K. pneumoniae* resistant to
193 marbofloxacin at the end of the trial, neither in the lungs of animals infected with the low *K.*
194 *pneumoniae* inoculum (groups A1 and A2) or in those infected with the large inoculum
195 (groups B1 and B2). Theses results differ from our previous results showing the emergence
196 of *K. pneumoniae* resistant to 0.3 µg/mL marbofloxacin in the animals infected by a large
197 inoculum and treated with 64 mg/kg of marbofloxacin (8). However, the present study was
198 longer and we observed rat mortality within the large inoculum group (groups B) during the
199 64 mg/kg marbofloxacin treatment, in contrast to the previous study. This mortality was
200 probably due to a higher sensitivity to the infection of gnotobiotic animals compared to
201 conventional rats. The animals that died during the treatment were possibly carriers of
202 resistant *K. pneumoniae* in the lungs (data not checked).
203
204 **Effect of different marbofloxacin dosage regimens on faecal microflora.**
205 For all animals, whatever the dosage regimen, the *E. faecium* population remained
206 unchanged during and after the marbofloxacin treatment (Figure 1, panel A). Unfortunately,
207 an evaluation of a resistant *E. faecium* population was not carried out due to an interaction
208 between marbofloxacin and Slanetz and Bartley culture medium.
209 For the *E. coli* population a decrease in the number of isolates was observed during the
210 treatment in all groups depending on both the marbofloxacin dose and the dosing regimen.
211 This decrease was followed by an increase to pre-treatment levels after termination of the
212 therapy (Figure 1, panel B). Seven days after the initiation of marbofloxacin treatment, a
213 resistant *E. coli* subpopulation (MIC ranging from 0.256 to 2 µg/mL) was found in all
214 treatment groups. The percentage of rats harbouring resistant intestinal *E. coli* was between
215 10 and 17% for groups A1, A2 and B2, and only the group B1 (high dose, single shot)
216 showed a higher percentage at 50% (Table 3). The fact that the amount of marbofloxacin
217 excreted in the faeces was approximately the same in the two groups (groups A and B) could

218 explain why the numbers of animals with resistant *E. coli* in their faeces were rather similar
219 for the different groups whatever the marbofloxacin total dose and regimens (single or
220 fractionated administrations, see Table 3). Nevertheless it was difficult to correlate the
221 emergence of resistant *E. coli* with the marbofloxacin concentration in the faeces since
222 marbofloxacin could be highly bound to faecal matter (12).
223 However, the most important result of this study was the intestinal colonization by the
224 targeted pathogenic bacteria, *K. pneumoniae,* present at the infection site. This *K.*
225 *pneumoniae* colonization was established throughout the study and seven days after the
226 start of antimicrobial treatment, it was observed in the majority of surviving animals for the
227 groups treated with a single administration (60% and 100% for the low and the high dose
228 respectively) (Table 3 and Figure 1, panel C). Deglutition by the infected animals of *K.*
229 *pneumoniae* moving out of the lung to the mucus escalator was probably the cause of the
230 colonization of the intestinal tract by *K. pneumoniae.*
231 More importantly, intestinal colonization by *K. pneumoniae* was accompanied by the
232 emergence in the gut of a *K. pneumoniae* subpopulation resistant to the fluoroquinolone.
233 Faecal *K. pneumoniae* resistant to 0.3 µg/mL marbofloxacin (MIC ranging from 0.512 to
234 2 µg/mL) were detected in 57% (8/14) of rats receiving a single dose (16 or 64 mg/kg)
235 whatever the dose level (Table 3). For animals receiving the fractionated marbofloxacin
236 administration, only one out of 14 rats carried a resistant *K. pneumoniae* subpopulation in the
237 gut (Table 3). It is noteworthy that emergence of resistant *K. pneumoniae* in the intestinal
238 tract occurred in animals harbouring no resistant *K. pneumoniae* in their lungs. This intestinal
239 colonization by resistant *K. pneumoniae* occurred in 54% of all surviving rats (15/28) and
240 seemed to be slightly more frequent in groups infected with the high inoculum (8/10 i.e. 80%,
241 Table 3) than with the low inoculum (7/18 i.e. 39%, Table 3). The most likely reason is that
242 the probability of colonizing the intestinal tract increases with the inoculum size in the lung
243 (more *K. pneumoniae* and more mucus production).
244 Furthermore, whatever the size of *K. pneumoniae* pulmonary inoculum, we observed a
245 higher percentage of intestinal colonization by resistant *K. pneumoniae* when the treatment

was a single dose administration of marbofloxacin rather that the fractionated administration of the same total dose (8/14 i.e. 57% for single vs. 1/14 i.e. 7% for fractionated, Table 3). In a previous study, we showed that the antibiotic exposure played an important role in the selection of resistant bacteria at the infection site (8), and in agreement with the present results, we also showed that the plasma antibiotic exposure with fractionated administration limited the enrichment of the resistant subpopulation compared with the single administration of the same total marbofloxacin dose.

However the design of the present study did not enable us to document whether the emergence of the resistant *K. pneumoniae* subpopulation occurred in the digestive tract or in the lungs, but some clues suggest the emergence of resistance in the gut. Indeed, we observed resistant *K. pneumoniae* in the gut of animals harbouring no resistant *K. pneumoniae* in their lungs. Moreover, for animals infected with the low inoculum and treated by the single administration of 16 mg/kg of marbofloxacin, the size of the *K. pneumoniae* population in the gut was higher than the population inoculated into the lungs (8.1 ± 1.1 $\log_{10}$ CFU/g of faeces vs. 5 $\log_{10}$ CFU/lung, Table 3), increasing the likelihood of resistant mutants appearing in this intestinal bacterial population. Therefore the scenario of the intestinal colonization by wild type *K. pneumoniae* followed by the appearance in the gut of resistant *K. pneumoniae* mutants secondarily selected by marbofloxacin intestinal exposure seems more probable than the gut colonization by resistant *K. pneumoniae* of pulmonary origin.

We are not in a position to estimate the exact antibiotic exposure in the gut; but it is likely that the dosing regimens that are optimised to achieve clinical success while minimising the emergence of resistance at the lung infection site may actually be inappropriate in terms of gut colonization and intestine-located emergence of resistance.

Conclusion.

For the sake of simplicity, the present study was carried out with an artificial intestinal ecosystem in di-associated gnotobiotic rats, i.e. a system lacking anaerobes that can play an

important role in colonization resistance (20). However, this animal model appears to be relevant to the study of intestinal colonization by resistant bacteria given that anaerobic flora could be decreased in patients by some antimicrobial treatments. Therefore, such a model represents an alternative to classical models of intestinal colonization by resistant pathogenic bacteria, where anaerobic flora are eradicated by clindamycin treatment before colonization challenge (6, 15). To our knowledge, this study is the first using a model of natural gut colonization by pathogenic bacteria from a non-intestinal infection site, and showing the emergence of resistant strains in this new intestinal population during fluoroquinolone treatment of a lung infection. Indeed, usually studies on intestinal colonization use orogastric gavage of bacteria already resistant to the antibiotic (6, 14).

In conclusion, the results of the present study highlight that a fluoroquinolone treatment leading to the bacterial cure and prevention of resistance emerging at the infection site might at the same time lead to the emergence of resistant pathogenic bacteria in the digestive tract secondary to intestinal colonization by wild type pathogens. This could generate a reservoir of resistant pathogens for secondary infections (16, 23). In addition it was shown that such an event is influenced, and thus might be manageable, by the dosage regimen. This is a difficult challenge because antibiotic exposure to prevent the emergence of resistance in a pathogen should not only apply to the target site of infection but should be extended to the gut flora when colonization occurs.

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TABLE 1

Amount of marbofloxacin excreted in the faeces of rats infected with a low or a high inoculum.

Marbofloxacin dose (mg/kg)	Marbofloxacin administered (µg, mean ± SD)	Amount of marbofloxacin excreted in faeces (µg, mean ± SD)	
		Low inoculum Group A	High inoculum Group B
4	835 ± 58	129 ± 24	42 ±23
16	3605 ± 232	896 ±116	168 ± 126
100	22650 ±636	nd	1340 ± 547

nd: not determined

TABLE 2.

Amount of *K. pneumoniae* in each rat lung and proportion of animals with resistant *K. pneumoniae* at the end of the experiment for the low (Group A) and the high (Group B) inocula.

Total marbofloxacin dose	**Low inoculum**		**High inoculum**	
	16 mg/kg		64 mg/kg	
regimen Animal group	Single A1	Fractionated A2	Single B1	Fractionated B2
n	10	8	7	7
No of dead animals	0	0	3	1
No of rats with *K. pneumoniae* in lungs at the end of experiment	1	1	4	6
total log10 cfu/lungs (mean +/- SD)	0.1 ± 0.2 [b]	0.3 ± 0.8 [b]	5.7 ± 2.0 [a]	5.9 ± 1.8 [a]
No of rats with resistant *K. pneumoniae* in lungs at the end of experiment	0	0	0 [a]	0 [a]

[a] Dead rats are not taken into consideration to calculate the total bacterial population in lungs

[b] For the calculation of these means, we assigned the value 0 $\log_{10}$ CFU to the lungs in which bacteria were undetectable.

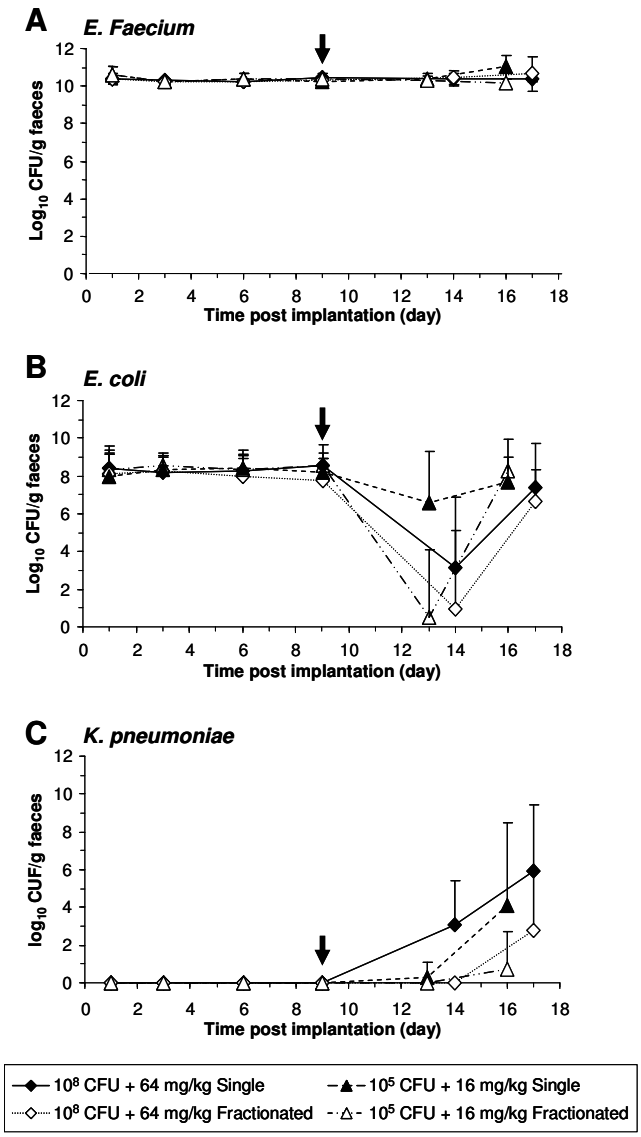
TABLE 3.

Percentage of surviving rats with the intestinal tract colonized by *E. coli* resistant to marbofloxacin, wild-type *K. pneumoniae* or *K. pneumoniae* resistant to marbofloxacin and amount of each population in faeces 4 and 7 days after the start of marbofloxacin treatment.

			4 days after initiation of marbofloxacin treatment						**7 days after initiation of marbofloxacin treatment**					
			***E. coli* Resistant**		***K. pneumoniae* Total**		***K. pneumoniae* Resistant**		***E. coli* Resistant**		***K. pneumoniae* Total**		***K. pneumoniae* Resistant**	
Inoculum size	Group	Total marbofloxacin dose and dosing regimen	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)	No. of animals with bacteria in faeces/ total no. animals	$\log_{10}$ CFU/g of faeces (mean ± SD)
LOW	**A1**	16 mg/kg - Single	0/10 (0%)	-	2/10 (20%)	5.0 ± 3.2	0/10 (0%)	-	1/10 (10%)	3.8 [a]	6/10 (60%)	8.1 ± 1.1	4/10 (40%)	3.8 ± 1.5
	A2	16 mg/kg - Fractionated	0/8 (0%)	-	0/8 (0%)	-	0/8 (0%)	-	1/8 (13%)	3.9 [a]	1/8 (13%)	5.7*	0/8 (0%)	-
HIGH	**B1**	64 mg/kg - Single	0/6 (0%)	-	5/6 (83%)	3.1 ± 2.4	3/6 (50%)	1.4 ± 1.9	2/4 (50%)	2.0 ± 0	4/4 (100%)	7.3 ± 1.3	4/4 (100%)	5.4 ± 0.1
	B2	64 mg/kg - Fractionated	0/6 (0%)	-	1/6 (17%)	1.7*	0/6 (0%)	-	1/6 (17%)	6.0 [a]	4/6 (67%)	5.9 ± 0.6	1/6 (17%)	1.7 [a]

[a] Only one animal of this group had bacteria in the faeces

317 **Figure 1.** Time course evolution of the levels of the total *E. faecium* (A), *E. coli* (B) and *K.*
318 *pneumoniae* (C) populations in the faeces of rats (Mean ± SD) before, during and after
319 marbofloxacin treatment. The arrow shows the day of the lung infection.
320



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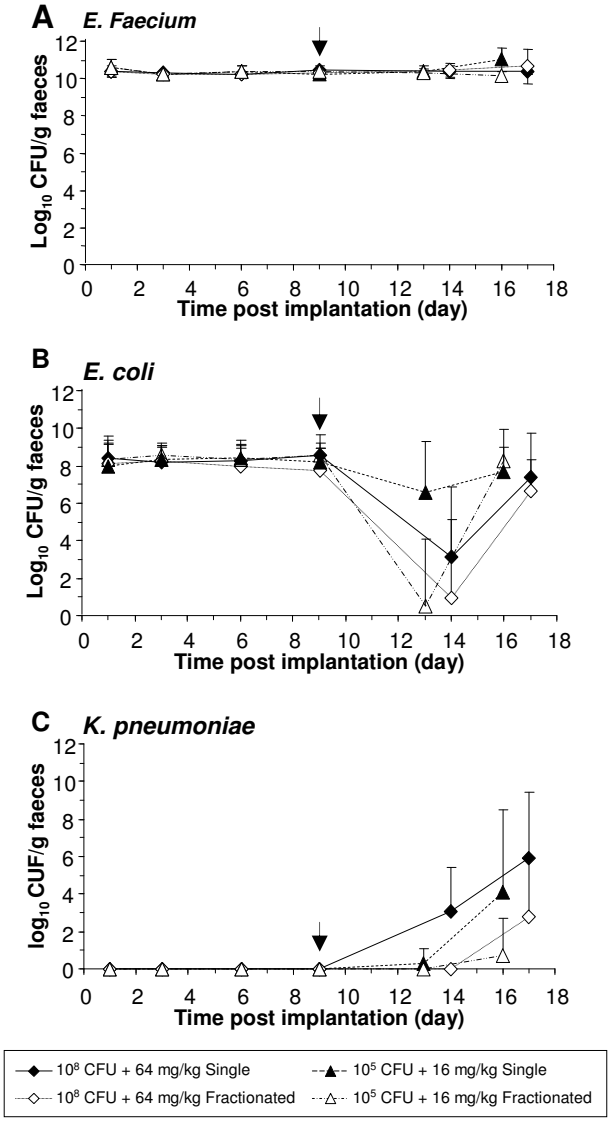
A
E. Faecium
Log10 CFU/g faeces
Time post implantation (day)
B
E. coli
Log10 CFU/g faeces
Time post implantation (day)
C
K. pneumoniae
log10 CUF/g faeces
Time post implantation (day)
10^8 CFU + 64 mg/kg Single
10^5 CFU + 16 mg/kg Single
10^8 CFU + 64 mg/kg Fractionated
10^5 CFU + 16 mg/kg Fractionated