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Evolution of fluoroquinolone-resistant *Escherichia coli* in the gut after ciprofloxacin treatment

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ABSTRACT

Background: Three healthy volunteers carried similar quinolone-resistant *E. coli* (QREC) (pulsed field gel electrophoresis profiles) in their gut before and after 14 days ciprofloxacin treatment. Given the intensity of the selective pressure and the mutagenic properties of quinolones, we determined whether these strains had evolved at the phenotypic and/or genomic levels.

Material and methods: Commensal QREC from before day-0 (D0), and a month after 14 days of ciprofloxacin (D42) were compared in 3 volunteers. Growth experiments were performed; acetate levels, mutation frequencies, quinolone MICs and antibiotic tolerance were measured at D0 and D42. Genomes were sequenced and single nucleotide polymorphisms (SNPs) between D0 and D42 were analyzed using DiscoSNP and breseq methods. Cytoplasmic proteins were extracted, HPLC performed and proteins identified using X!tandem software; abundances were measured by mass spectrometry using the Spectral Counting (SC) and eXtraction Ion Chromatograms (XIC) integration methods.

Results: No difference was found in MICs, growth characteristics, acetate concentrations, mutation frequencies, tolerance profiles, phylogroups, O-and H-types, *fimH* alleles and sequence types between D0 and D42. No SNP variation was evidenced between D0 and D42 isolates for 2/3 subjects; 2 SNP variations were evidenced in one. At the protein level, very few significant protein abundance differences were identified between D0 and D42.

Conclusion: No fitness, tolerance, metabolic or genomic evolution of commensal QREC was observed overtime, despite massive exposure to ciprofloxacin in the gut. The three strains behaved as if they had been unaffected by ciprofloxacin, suggesting that gut may act as a sanctuary where bacteria would be protected from the effect of antibiotics and survive without any detrimental effect of stress.

1. Introduction

Fluoroquinolones (FQ) are among the most prescribed antibiotics in the world because they are highly bactericidal, have a satisfactory tolerance profile, high intracellular and tissue penetration and an excellent oral bioavailability (Hooper, 2001; Schentag, 2000). Unfortunately, a major increase in FQ-resistance both in the hospital-setting and the community has occurred, responsible for clinical failures and increase in the use of other broad-spectrum antibiotics which further promotes the emergence of multidrug resistant (MDR) bacteria (Davidson et al., 2002; Nseir et al., 2005; Weber et al., 2003). The rapid emergence of resistance is in part due to very high concentrations of FQ found in the gut, with consequently a major impact of FQ on commensals such as *Escherichia coli,* which happens to also be a major pathogen (Beyer et al., 2000; Brumfitt et al., 1984; Darouiche et al., 1990; de Lastours and Fantin, 2015; Fantin et al., 2009; Pecquet et al., 1990).

The accumulation of FQ resistance mechanisms is associated with a fitness cost in *E. coli* (Andersson, 2006; Komp Lindgren et al., 2005; Marcusson et al., 2009). Therefore, once the selective pressure from the antibiotic treatment is lifted, one would expect FQ-resistant strains selected to be cleared from the gut. However, in a previous work from our group, 14/48 healthy subjects exposed to ciprofloxacin (cipro)

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carried quinolone-resistant *E. coli* (QREC) four weeks after the end of treatment, when no more cipro was detectable in the stools (Fantin et al., 2009). These resistant strains were able to persist in the gut in the absence of cipro, because they were highly adapted to commensal lifestyle, thanks to high growth rates and to increased capacities to resist stress and capture iron (de Lastours et al., 2012, 2014). No selection of resistant mutants from the initial microbiota was evidenced as no parental strain could be identified from the initial gut microbiota, and therefore an exogenous acquisition of QREC strains was considered as the most likely phenomenon (de Lastours et al., 2012). In contrast however, we found that the QREC present before cipro treatment in 4/14 subjects had identical pulsed field gel electrophoresis (PFGE) profiles than those collected after the end of treatment, suggesting that the same QREC strains persisted overtime throughout cipro treatment (de Lastours et al., 2012). Antibiotic tolerance may explain this phenomenon; indeed, tolerance allows bacteria to temporarily survive longer in presence of lethal doses of bactericidal antibiotics but without being able to grow in their presence (Balaban et al., 2019).

This particular finding, using FQ resistance as a marker, gave the opportunity to follow the evolution of these strains in their natural environment during a 6 week-period including 2 weeks of major antibiotic exposure. Our aim was to determine whether these strains had evolved (i) in terms of an integrated phenotype measuring growth in various conditions, mutation frequencies and antibiotic tolerance; (ii) at the genome level; and/or (iii) at the protein level. Given the intensity of the selective pressure associated to the known mutagenic properties of FQ, we hypothesized that genetic and/or metabolic modifications had occurred among surviving resistant strains (Baharoglu and Mazel, 2011; Soto et al., 2006; Yim et al., 2011).

2. Methods

2.1. Clinical study and strain characteristics

The CIPHARES study was a prospective randomized controlled study involving 48 healthy adult volunteers who received six different dosage regimen of cipro for 14 days (Fantin et al., 2009). Among them, four volunteers carried QREC before and after cipro treatment; the isolates from before and after treatment were similar according to random amplification of polymorphic DNA (RAPD) and PFGE profiles (de Lastours et al., 2012). However, after phylogrouping by PCR, one subject had QREC from day 0 and day 42 that belonged to different phylogroups; QREC were finally similar only in 3 of the 4 subjects (V44, V45 and V48) which are the subject of this work."

We considered isolates before treatment (D0) and during and/or after cipro (D42 for V44 and V45 and D8, D14 and D42 for V48) (Table 1). Strains from V44 and V45 expressed low-level resistance to FQ (cipro minimum inhibitory concentration -MIC: 0.25–0.5 mg/L), whereas strains from V48 expressed high-level resistance to FQ (cipro MIC=32 mg/L), in accordance with the mutations identified in *par* and *gyr* genes (Table 1). All quinolone MICs had been determined previously by the broth dilution method (de Lastours et al., 2012; Fantin et al., 2009) and were measured again here and remained unchanged, except for strain V44 on D42 for which nalidixic acid MIC had initially been measured at 64 mg/L and we found here 512 mg/L, while cipro MIC remained the same (0.25 mg/L). Median concentrations of cipro in stool specimens at steady state (D7, D14) were 845 mg/L (range, 455–3235 mg/L); cipro was undetectable in all stool specimens at D42 (Fantin et al., 2009).

2.2. In vitro growth experiments

Growth experiments were performed in complex nutrient broth (NB) medium supplemented with 20 mM of glucose or gluconate and in phosphate minimal medium supplemented with 20 mM of glucose or gluconate (Bouvet et al., 2017), by OD measurements up to 24 hours of growth. Glucose was chosen, as it is the standard carbon source for *E. coli,* whereas gluconate is one of the major carbon sources for *E. coli* in the intestine (Chang et al., 2004). Cells preserved at −80 °C were grown overnight at 37 °C in lysogeny broth (LB), centrifuged and washed three times in phosphate buffered saline (PBS) before re-suspension in the adequate medium. Experiments were assayed using 96-well plates at 37 °C and OD_{600} was measured with a Tecan Infinite M200® plate reader. Each growth curve was characterized by three parameters: the lag time in mn (the time taken to reach the maximum growth rate), the maximum growth rate (MGR) in h^{-1} (the doubling time in mn being 60/MGR) and the biomass corresponding to the OD_{600} in stationary phase after 24 h of growth. We checked for the three couples of strains that growth curves obtained by OD measurements were similar to those obtained by colony counts in NB glucose medium (data not shown). Acetate concentrations remaining in the culture supernatants after 24 h of growth in minimal medium with glucose were measured (Bouvet et al., 2017). Acetate levels were used as a proxy of central metabolism as acetate is both produced and consumed during growth on glucose (Enjalbert et al., 2017; Wolfe, 2005).

Mutation frequencies of the low-level quinolone resistant isolates were estimated by monitoring their capacity to generate mutations conferring resistance to rifampicin (non-lethal mutations in the *rpoB* gene), as described previously (Denamur et al., 2002). *E. coli* M13 (mutator with a large *mutS* deletion) and K-12 MG1655 (non-mutator) strains were included as controls in each experiment. Mutagenesis was assessed with and without cipro. The tested concentration of the antibiotic was 0.25xMIC of the strains (0.0625 mg/ml for V44 isolates, 0.125 mg/ml for V45 isolates and 0.0039 mg/ml for controls).

Antibiotic tolerance experiments were conducted at 37 °C in Muller Hinton (MH). Overnight cultures were diluted 100-fold in 10 mL of fresh medium in 50 mL polypropylene tubes and incubated for 2 hours with shaking. To distinguish between tolerance/persistence and other modes of resistance, we chose to perform the killing curves at high ciprofloxacin concentrations (10 x MIC, i. e. 2.5 mg/L and 5 mg/L). Cipro was added and cells were serially diluted and plated on MH agar plates at different time points (t0h just before Cipro treatment, t1h, t2h, t3h, t4h, t6h and t24h) as well as additional growth controls without ciprofloxacin. We measured the survival fraction at 24 hours to make sure there was no growth of a resistant or quiescent sub-population.

All experiments were repeated 3 times. Data by strain and media

Table 1
Phenotypic and genetic characteristics of the quinolone-resistant *E. coli* strains. MIC: minimum inhibitory concentration.

Strain ID	MIC mg/L		*gyrA*	*gyrB*	*parC*	*parE*
	Ciprofloxacin	Nalidixic acid				
$V44_{D0}$	0.25	512	S83L, D678E	S492N	D475E	No mutation
$V44_{D42}$	0.25	512	S83L, D678E	S492N	D475E	No mutation
$V45_{D0}$	0.5	> 1024	S83L, D678E	E703D	E84G	No mutation
$V45_{D42}$	0.5	> 1024	S83L, D678E	E703D	E84G	No mutation
$V48_{D0}$	32	> 1024	S83L, D87N, A828S	A618T	S80I, E84V	V136I, I529L
$V48_{D8}$	32	> 1024	S83L, D87N, A828S	A618T	S80I, E84V	V136I, I529L
$V48_{D14}$	64	> 1024	S83L, D87N, A828S	A618T	S80I, E84V	V136I, I529L
$V48_{D42}$	64	> 1024	S83L, D87N, A828S	A618T	S80I, E84V	V136I, I529L

were compared using a Welch test or Mann-Whitney rank sum test.

2.3. Genomic comparison

Total DNA was extracted from one colony of D0 and D42 strains grown on LB plate using Wizard® genomic DNA purification kit (Promega®). Genomes were then sequenced using the Illumina HiSeq® with an average of almost 22 million paired-end reads of 150 base-pair per read. Assembly was performed with Velvet on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) and annotation was performed at the MicroScope platform (http://www.genoscope.cns.fr/agc/microscope) (Vallenet et al., 2017). The sequences have been deposited in the European Nucleotide Archive. Phylogroup assignation was performed with the ClermonTyper (Beghain et al., 2018). O-and H-types, *fimH* allele, multilocus sequence type (MLST) determination and resistome were performed on the CGE platform. To determine whether plasmid content was different between D0 and D42, we searched for replicons using the CGE and plasmidic sequences using PlaScope (Royer et al., 2018).

We also searched for virulence genes/alleles among a set of 20 virulence factors characteristic of extraintestinal pathogen *E. coli* (Clermont et al., 2017). Single nucleotide polymorphisms (SNPs) between each D0 and D42 strain genome pair were analyzed using two separate methods. First, DiscoSNP, a reference free based algorithm to find the SNPs, was used, the SNPs with a poor quality (rank >0.5) being filtered out (Uricaru et al., 2015). Second, breseq was used (Deatherage and Barrick, 2014). The impact on the protein of specific non-synonymous SNPs were determined using the SIFT tool (Kumar et al., 2009).

2.4. Proteomic analysis

Twenty µL of an overnight culture in LB were used to inoculate 200 mL of NB medium supplemented with 20 mM of gluconate in a 1 L flask at 37 °C. Culture was stopped once the OD reached 0.1 at 600 nm, which corresponds to the mid-exponential phase. The culture was then centrifuged, washed twice in PBS and the pellet was frozen at −80 °C. Three independent cultures for each D0 and D42 isolates from the three volunteers were performed and further analyzed. Cytoplasmic proteins were enriched as previously described (Rul et al., 2011), and liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the PAPPSO platform, (INRA, France). Using the PAPPSO tool suite (http://pappso.inra.fr/bioinfo), proteins were identified by querying *E. coli* protein databases (V44 day 0: 31940 sequences; V45 Day 0: 31220 sequences; V48 day 0: 32187 sequences) associated to a classical proteomic contaminant database. Spectral Count (SC) and eXtraction Ion Chromatograms (XIC) data were used for protein quantification and comparative statistics (see supplemental data for details on proteomic analysis).

The mass spectrometry proteomics data have been deposited on PROTICdb platform: https://clicktime.symantec.com/3YbiAQQGT9M1YgheV5VTmBr6H2?u=http%3A%2F%2Fmoulon.inra.fr%2Fprotic%2Fqrecevol.

3. Results

3.1. Growth experiments

No difference was evidenced in terms of growth, fitness (as measured by the MGR) of the strains in various media reflecting different carbon sources (Table 2), lag time or biomass (data not shown) when D0 and D42 isolates from the same volunteer were compared, whichever medium was used. Furthermore, for V48, isolates from D8 and D14 exhibited similar growth characteristics to D0/D42 isolates. No difference was observed in the acetate concentrations measured in minimum medium supplemented with glucose after 24 h of growth between D0 and D42 isolates (Table 2). In summary, the main metabolic characteristics of the strains before and after cipro treatment remained unchanged.

3.2. Mutation frequencies

The strains were not found to be hypermutators. Although not significant for all strains, a slight increase in rifampicin mutation frequency in the presence of sub-inhibitory concentrations of cipro was observed for controls and isolates from volunteers V44 and V45 (which have low-level resistance to fluoroquinolones), in accordance with the known effect on mutagenesis of FQ. However, no difference was observed in basal or induced mutagenesis between isolates of a same volunteer (Fig. 1).

3.3. Antibiotic tolerance

After performing time-kill experiments over 24 h in presence of

Table 2
Main genomic characteristics and metabolic properties of the *E. coli* strains. ST: Sequence type, IP: Institut Pasteur, MGR: Maximum growth rate, NB, Nutrient broth, MM: Minimum medium, ND: not determined * Number of genes/alleles detected over the 20 searched (Clermont et al., 2017).

Strain ID	Phylogroup	ST		Serotype	*fimH*	Resistome (except quinolone)	Virulome	MGR (h^{-1})				Acetate
		Warwick	IP		allele		(n*)	NB Glucose (s.d.)	NB Gluconate (s.d.)	MM Glucose (s.d.)	MM Gluconate (s.d.)	(nM)
$V44_{D0}$	G	117	48	O24:H4	*H97*	bla_{TEM-1}, *tet(A)*	6	1.38 (0.05)	1.21 (0.11)	0.70 (0.06)	0.67 (0.02)	12.8 (1.3)
$V44_{D42}$	G	117	48	O24:H4	*H97*	bla_{TEM-1}, *tet(A)*	6	1.34 (0.05)	1.27 (0.12)	0.70 (0.04)	0.69 (0.02)	13.3 (2.5)
$V45_{D0}$	D	69	3	O17:H19	*H27*	bla_{TEM-1}, *sul2, dfrA17, tet(B)*	7	1.32 (0.05)	1.22 (0.07)	0.71 (0.06)	0.62 (0.01)	14.1 (3.3)
$V45_{D42}$	D	69	3	O17:H19	*H27*	bla_{TEM-1}, *sul2, dfrA17, tet(B)*	7	1.35 (0.02)	1.25 (0.10)	0.65 (0.01)	0.53 (0.04)	13.9 (3.7)
$V48_{D0}$	B2	131	43	O25b:H4	*H30*	bla_{TEM-1}	8	1.31 (0.05)	1.22 (0.06)	0.69 (0.04)	0.65 (0.07)	17.5 (2.8)
$V48_{D8}$	B2	131	43	O25b:H4	ND	ND	ND	1.31 (0.05)	1.22 (0.06)	0.69 (0.03)	0.68 (0.02)	ND
$V48_{D14}$	B2	131	43	O25b:H4	ND	ND	ND	1.31 (0.05)	1.22 (0.06)	0.71 (0.02)	0.66 (0.02)	ND
$V48_{D42}$	B2	131	43	O25b:H4	*H30*	bla_{TEM-1}	8	1.30 (0.05)	1.20 (0.02)	0.67 (0.03)	0.72 (0.01)	16.5 (3.8)

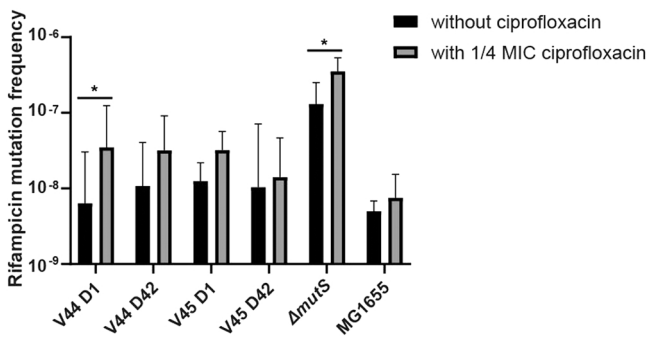

Fig. 1. Mutation frequencies of the low-level quinolone resistant *E. coli* isolates from the V44 and V45 volunteers with and without ciprofloxacin, The y-axis represents the frequency of rifampicin resistance acquisition expressed as the median of at least nine values with the interquartile range. *E. coli* M13 and K-12 MG1655 are respectively mutator (*mutS* deleted) and non-mutator controls. Ciprofloxacin was used at 0.25x MIC. *P < 0.05; Mann-Whitney rank sum test.

2.5 mg/L and 5 mg/L Cipro as well as controls with no Cipro, we found that although the slopes of the killing curves slightly differed between the two strains isolated from the volunteers 44 and 45, there was no difference seen in their tolerance between day 0 and day 42, and no regrowth at 24 h (Fig. 2).

3.4. Genomic analysis

Whole genome sequencing showed that phylogroups, O-and H-types, *fimH* alleles and sequence types were similar on D0 and D42 for each couple of strain (Table 2), confirming our first line of typing using RAPD and PFGE-profiles. All three subjects were colonized with QREC belonging to epidemic clones that have been reported to carry multiple antibiotic resistances (ST117-G, ST69-CGA-D and ST131-B2) (Clermont et al., 2008, 2019; Johnson et al., 2011). The QRDR mutations identified confirmed classical Sanger sequencing findings (Table 1). Genes coding for resistance to other antibiotics were similar between isolates from D0 and D42. All strains possessed a bla_{TEM-1} gene conferring resistance to ampicillin, strains from V44 and V45 harboured *tet* genes and isolates from V45 had genes conferring resistance to cotrimoxazole. Virulence genes were also found similarly in D0 and D42 isolates; all QREC carried extra-intestinal virulence genes (Tables 2 and S1) with an increasing gradient from G strain to B2 strain, in accordance with the link between phylogeny and virulence. Of note, all the strains possessed the high pathogenicity island (HPI) genes (*fyuA, irp2*) involved in iron capture. As expected, each strain has one to two large IncF plasmids and several small Col plasmids. One strain, V45 has in addition an IncX1 plasmid. Very few differences were observed between D0 and D42 isolates with the mobility of small Col plasmids corresponding to less than 10% of the plasmid sequences (Table S2). No SNP was evidenced between D0 and D42 isolates for V44 and V48 with either method used. Concerning V45, two SNPs were evidenced at D42 with both DiscoSNP and breseq methods, a synonymous A->G mutation (Q314Q) in *mdtE* coding for a multidrug resistance protein and a non-synonymous A->T mutation (S362C) in *mdoB* coding for a phosphotransferase, considered to be tolerated according to SIFT (score of 0.07) (Kumar et al., 2009). In summary, the strains isolated in the three volunteers belong to epidemic resistant clones and no significant genetic modification occurred during the 42 days spent in the gut despite high concentrations of cipro.

3.5. Comparative proteomics analysis

The total number of proteins identified was 1540, 1764 and 1563 for the V44, V45 and V48 isolates, respectively. Using the XIC integration method, no difference was found between isolates before and after cipro treatment from each of the 3 subjects. Using the SC method, no difference was observed for V44 isolates whereas one and two different protein abundances were evidenced for V48 and V45 isolates, respectively. For V48, a significant decrease by 3-fold in the abundance of MetH, a methionine synthase, was found between D0 and D42 (Fig. S1). For V45, the protein abundances of LpxL, a lipid A biosynthesis lauroyltransferase and MtlA, a component of a phosphoenolpyruvate-dependent sugar phosphotransferase system, were significantly decreased between D0 and D42 (4 and 2-fold, respectively, Fig. S2) (Jacobson et al., 1983, 1979). Altogether, very few significant protein abundance differences were identified only with the SC method, indicating that the proteomes did not undergo major changes between D0 and D42.

4. Discussion

This work reports the fate of a unique collection of quinolone-resistant commensal *E. coli* strains that survived for 42 days in the gut of healthy volunteers despite exposure to massive cipro concentrations in stool, far above the MIC of cipro against these strains. Using *in vitro* growth and tolerance assays, genomic and proteomic tools, we found no fitness, metabolic or genetic evolution of these strains overtime. The level of SNPs that we observed in the strains is compatible with a neutral evolution observed in a dominant *E. coli* clone during a one-year period in a healthy subject that did not take any antibiotics. This suggests that the strains are well adapted to their environment, with a number of generations per day estimated in the healthy gut between 1 and 6 (Ghalayini et al., 2018). The proteome analysis did not evidence modulation of expression after FQ exposure, contrary to what has been observed *in vitro* in *Salmonella* spp, where the conditions of contacts between bacteria and FQ are well controlled (Yim et al., 2011). In summary, the three strains behaved as if they were unaffected by cipro

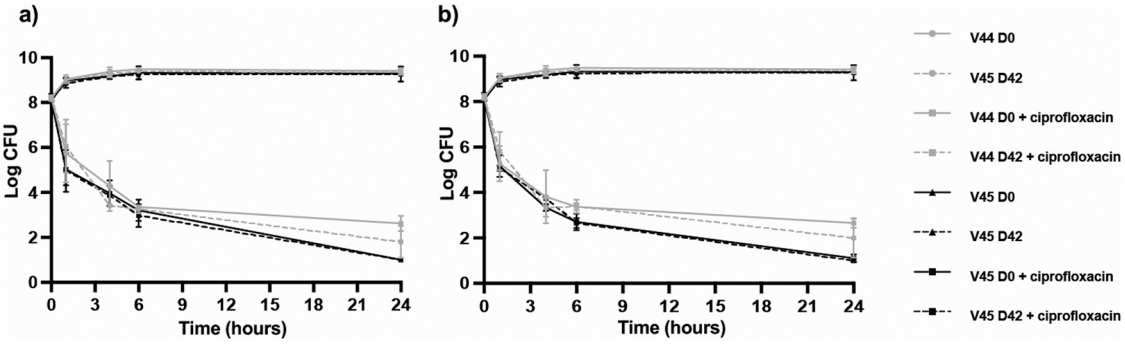

Fig. 2. Time-dependent killing curves (tolerance profiles) of the low-level quinolone resistant *E. coli* isolates from the V44 and V45 volunteers. Survival fraction (CFUs) without and with ciprofloxacin challenge: a) 2.5 mg/L; b) 5 mg/L of the V44 and the V45 volunteers' strains collected at days 1 and 42. Error bars show standard deviation of three biological replicates.

treatment. These results are surprising, especially given that quinolones have well demonstrated mutagenic properties in several species (Soto et al., 2006). Indeed, in *E. coli*, quinolones induce partial or total loss of PAIs *in vitro* by SOS-dependent or -independent pathways; however all strains here carried PAIs which were unaffected by cipro exposure (Soto et al., 2006). Cipro has been found to induce a two-to-four fold increase in mutagenesis of *E. coli* K-12 (Baharoglu and Mazel, 2011; Thi et al., 2011), by inactivating the gyrase which leads to DNA breaks, inducing SOS and mutagenesis (Baharoglu and Mazel, 2011). However, despite the presence of gyrase mutations perturbing quinolone binding, we observed a slight increase in mutagenesis of *E. coli* exposed to cipro but without differences between D0 and D42 isolates. This mutagenesis increase did not translate in SNP variations in the genomes, also suggesting a limited effect of the quinolone. Furthermore, the cipro-MIC of the strains as well as the genetic support of resistance remained unchanged between D0 and D42. The absence of significant SNP for quinolones resistance between D0 and D42 further confirms that no resistance mechanism such as mutations in marR, soxR, or acrR, was selected (Garoff et al., 2018). As the quinolone resistance of the strains did not change, we were interested in measuring their tolerance to cipro. The absence of difference in the tolerance profile after cipro treatment is in line with the absence of growth and metabolic differences observed after treatment. The addition of different methods converging to show the absence of evolution or mutation of these strains is a clear strength of this study.

The fact that QREC strains were not detected in two subjects during cipro treatment but detected again after the end of treatment is puzzling. These two strains exhibit low-level resistance to FQ and did not increase their MIC to FQ. The detection limit of our technique, estimated at 10^2/g of stool, may explain why these strains were not detected during treatment (de Lastours et al., 2012). However, given the extremely high cipro concentrations in the stools during treatment, they should have been eradicated and unable to persist after the end of treatment, as observed for susceptible Enterobacteriales. This suggests they may have been able to hide in some ecological niche in the gut, be protected by mucus or form a protective biofilm, allowing them to escape the bactericidal properties of cipro (Sorbara and Pamer, 2018). Also, measured cipro concentrations in the feces may not be directly translated into active drug as local conditions sharply differ between fecal environment and broth for MIC determination. Another hypothesis is that the cipro concentrations measured in the stools may differ at different levels of the gut microbiota in the colon and allow some *E. coli* to escape, and especially FQ-resistant ones which are favored in case of sub-optimal cipro concentrations. Once cipro concentrations decrease, they recolonize the gut. Finally, it is also possible that volunteers may have been recolonized after treatment (when ciprofloxacin concentrations decreased) with the same highly adapted QREC strain that persisted in their environment. Indeed, it has been shown that long-term persistence and multiple-host sharing of *E. coli* clones within household members occurs (Johnson et al., 2008).

Interestingly, these three strains which persisted despite exposure to cipro all have specific characteristics which may explain our results in part. Indeed, they belong to highly successful epidemic *E. coli* clones with multiple virulence genes, including iron capture systems (Clermont et al., 2019, 2008; Johnson et al., 2011). For one subject the QREC at the end of treatment was highly dominant as it was the only *E. coli* strain found, despite the absence of cipro at that time. We have previously shown that these strains have also high *in vitro* and *in vivo* fitness. All these characteristics reflect a high adaption to commensal lifestyle, which allow them to compete successfully against other bacteria in the gut microbiota (de Lastours et al., 2014).

In conclusion, despite a major stress induced by 14 days of cipro treatment, the FQ-resistant *E. coli* colonizing the gut of healthy volunteers did not evolve in anyway, in terms of neither quinolone resistance, fitness, tolerance, protein expression or genetic mutations. These findings highlight the difficulty to extrapolate data obtained *in vitro* to the complexity of the gut microbiome. They show that the gut may act as a sanctuary where bacteria can be protected from the effect of antibiotics and survive without any detrimental effect of stress. Simple models considering only the *in vitro* MIC and the dosage of antibiotics in stools will be clearly insufficient to explain the emergence and persistence of resistant strains in the gut.

Transparency declarations

All authors declare they have no conflict of interest involving this work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ijmm.2022.151548.

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