A murine model to study the gut bacteria parameters during complex antibiotics like cefotaxime and ceftriaxone treatment

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Title: A murine model to study the gut bacteria parameters during complex antibiotics like cefotaxime and ceftriaxone treatment.

Running title: Ceftriaxone increase intestinal carriage of ESBL-producing *Klebsiella*

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Abstract :

Background: The globally increasing resistance due to extended-spectrum beta-lactamase producing *Enterobacteriaceae* is a major concern. The objective of this work was to develop a murine model to study the gut bacteria parameters during complex antibiotics like cefotaxime and ceftriaxone treatment and to compare the fecal carriage of ESBL-producing *Enterobacteriaceae*.

Methods: SWISS mice were treated either with ceftriaxone or with cefotaxime or with NaCl 0.9% as a control group from day 1 to day 5. We performed a gavage at day 4 with a *Klebsiella pneumonia* CTX-M9. We collected stools and performed pharmacological

measurements, cultures and 16S rRNA gene amplification and sequencing during the 12 days of the stool collection.

Results: Mice treated with ceftriaxone were more colonized than mice treated with cefotaxime after gavage (p-value = 0.008; Kruskal-Wallis test). Ceftriaxone and cefotaxime were both excreted in large quantity in gut lumen but they drove architecture of the gut microbiota in different trajectories. Highest levels of colonization were associated with particular microbiota composition using principal coordinate analysis (PCoA) which were more often achieved in ceftriaxone-treated mice and which were preceded by highest fecal antibiotics concentrations in both cefotaxime or ceftriaxone groups. Using LEfSe, we found that twelve taxa were significantly different between cefotaxime and ceftriaxone-treated mice. Using SplinectomeR, we found that relative abundances of *Klebsiella* were significantly higher in CRO than in CTX-treated mice (p-value = 0.01).

Conclusion: Ceftriaxone selects a particular microbial community and its substitution of for cefotaxime could prevent the selection of extended-spectrum beta-lactamase producing *Enterobacteriaceae*.

Keywords: beta-lactamase, gastrointestinal microbiome, enterobacteriaceae, extended-spectrum beta-lactamase

Introduction

The increasing global resistance of *Enterobacteriaceae* to beta-lactams is a major public health concern for the years to come and led the World Health Organization to classify the resistance of *Enterobacteriaceae* to third-generation cephalosporins (3GC) as a critical priority for research and development of new antibiotics in February 2017 (1). The majority of 3GC-resistant *Enterobacteriaceae* produces extended-spectrum beta-lactamase (ESBL) which increases morbidity and mortality, length of stay and health costs (2,3).

The microbiota would be composed of 800 to 1000 species comprising more than 7000 different strains (4). Within this population of 10^{12} to 10^{13} individuals, bacteria are carrying genes for antibiotic resistance. Antibiotics conduct to a partial destruction of the gut bacteria (mainly anaerobic) susceptible to the given agent, thus leaving room and access to the necessary resources for organisms resistant to this treatment (5). Knowing that gut microbiota is a reservoir for ESBL-producing *Enterobacteriaceae*, their multiplication can lead to excretion in the external environment and inter-individual transmission (6,7). These germs may be responsible for infections that will be difficult to treat (8). One of the possibilities to limit the emergence of resistant mutants is to choose the antibiotics that have the lowest impact on the microbiota but knowledge that could drive this choice are still scarce.

Studies show that use of 3GC and exposure to 3GC favors ESBL-mediated resistance in *Enterobacteriaceae* (9–11). Although they have similar antibacterial spectrum, ceftriaxone (CRO) and cefotaxime (CTX) may have different impact on ESBL-mediated resistance. Specifically, it is advocated that CRO has more impact on the gut microbiota than CTX, due to higher biliary elimination than CTX (12). In a large multicentric study involving 701 health care facilities, use of CRO is positively associated with 3GC-resistant *E. coli*, whereas CTX use is not (13). However, use of both 3GC is associated with 3GC-resistant *Klebsiella. pneumonia* (*Kp*) in the same study, and with 3GC-resistant *E. cloacae* in another one (12,13).

The impact of replacing CRO by CTX on resistance to 3GC is reported in 2 hospital-level studies. In one center, it is associated with a slower growth of high-level cephalosporinase mediated resistance, mainly in *E. cloacae*, but has no impact on ESBL-mediated resistance (14). More recently, Tan *et al.* show that switching from CRO to CTX is associated with a decreased incidence of hospital-acquired infection caused by ESBL-producing *Enterobacteriaceae* (15). Lately, Burdet *et al*. do not found different effects on the microbiota in healthy volunteers treated 3 days with CRO or CTX (16). Hence, the ecological advantage of CTX on CRO remains debated, and no experimental study has compared the effects of CRO and CTX on the fecal microbiota.

The main objective of this experimental work was to develop a murine model to study the gut bacteria parameters during complex antibiotics like CTX and CRO treatment and to compare the fecal carriage of ESBL-producing *Enterobacteriaceae*.

Materials and Methods

Animals

SWISS non-consanguineous albino mice provided by the JANVIER laboratory (Le Genest-Saint-Isle, France) were used. These are robust and non-aggressive animals often used for pharmacological modeling and already used to study the carriage of multidrug-resistant *Enterobacteriaceae* (17). We used females aged 6 weeks and weighing between 25 and 30 g, guaranteed without pathogens. The mice were housed at the Animal Research Center of the Institute of Health Research 2, University of Nantes, in a controlled environment (day/night cycle of 12h, extinction 7:30 pm) with a controlled sterile diet. After a review of the literature on multidrug-resistant bacteria implantation in murine models, the number of mice per treatment group was set at 15 (18–21). In addition, mice were isolated in individual cages to

prevent cross-contamination by coprophagia. Drinking water and food were provided *ad libitum*. Study was approved by the French Ministry of Higher Education and Research (APAFIS 8528, Site agreement A44279, ethics committee 006).

Antibiotics

Compared with CTX structure, CRO has a basic 2-(2-amino-4-thiazolyl)-2-(Z)-methoxyimino-acetyl side chain which led to a very long elimination half-life of 8 hours in human, high beta-lactamase stability and extremely high chemotherapeutic efficacy against a broad spectrum of Gram-positive and Gram-negative pathogens. Therefore, in human, due to its extended half-life, CRO is used one or twice daily while CTX is used 3-time daily or in continuous infusion. Reconstitution of CRO and CTX was performed in physiological saline (NaCl 0.9%) to obtain initial concentrations of 100 g/L. Mice were randomized in three groups: Group 1 received CRO 250 mg/kg twice daily subcutaneously from day 1 to day 5, group 2 received CTX 500 mg/kg three times daily from day 1 to day 5 and the control group received NaCl 0.9% three times daily from day 1 to day 5. Taking into account the very short half-life of these molecules in mice and the concentrations usually found in humans, it was decided to use high dosages corresponding to 5 times the recommended dosage in humans when treating severe infections such as bacterial meningitis or infectious endocarditis (22–24). Indeed, we carried out a preliminary pharmacokinetic study to choose the optimal dosage to be adopted based on the plasma and fecal concentrations usually found in patients treated for infections. Maximum plasma concentration and mean plasma half-life of CTX (after 250 mg/kg, n = 5) in our mice were 207 mg/L and 24 minutes, respectively and maximum plasma concentration and mean plasma half-life of CRO (after 500 mg/kg, n = 1) were 264 mg/L and 78 minutes, respectively. Plasma concentrations were similar to those usually observed in human and concentrations of CRO and CTX in the feces were they similar to those observed

by previously published studies (279 µg/g and 167 µg/g respectively) (17,25,26). We therefore chose to administer 500 mg/kg three times a day for CTX and 250 mg/kg twice a day for CRO. All subcutaneous injections were 200 µL.

Bacterial strain and gavage

The strain used for gavage was a clinical isolate of *Kp*, carrying the beta-lactamase CTX-M9 (MIC of CTX: 512 mg/L, MIC of CRO: 2046 mg/L). The plasmids harboring most bla_{CTX-M} are transferable among bacterial cells, especially in the gut (27). Inocula of 10^5 CFU were prepared immediately before gavage using a spectrophotometer. Gavage was done at day 4 of the antibiotic treatment.

Three runs of 5 mice in each group (CRO, CTX and NaCl) were performed.

Stool collection and culture

Stool sampling was performed at day 1, 4, 6, 8, 10 and 12. On the day of sampling, each mouse was placed in a clean cage for 1 hour to collect his stool and was returned to his "accommodation" cage. Stools were immediately frozen at -80 ° C.

Each stool was weighed and then crushed (Ultraturrax Tube Drive®, IKA ™, Germany) with 10 µL saline per milligram of stool. Serial dilutions were performed in the fresh state, then manual inoculations of 100 µL on chromogenic media with mixture of antibiotics, including cefpodoxime (ChromID ESBL, BioMérieux ™, Marcy L'Etoile, France) were carried out before incubation at 37°C of 24h.

The detection threshold was 2 $\log_{10}$ CFU/g stool. At least one colony per culture medium was identified using mass spectrometry (MALDI TOF, Vitek MS®, Biomérieux ™, Marcy L'Etoile, France) to confirm genus and species.

16S rRNA gene amplification and sequencing

Fecal samples were kept frozen at −80 °C until they were processed. After fecal DNA isolation using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA fecal DNA kit) including an enzymatically cell lysis step, amplicons spanning the variable region 4 of bacterial 16S rRNA gene were generated and sequenced using Illumina Mi-seq platform at the University of Minnesota Genomic Center, Twin Cities, MN (28). The 16S rRNA gene sequencing data from the Illumina runs were trimmed and filtered using SHI7 (29). We then performed operational taxonomic units (OTUs) assignment using 'NINJA-OPS' against the Greengenes 13.8 database as a reference, by clustering the sequences with a threshold of 97% pairwise identity (30,31). Next, we used Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 for diversity analyses (32). We presented beta diversity, based on Unweighted UniFrac distances, a β-diversity measure that uses phylogenetic information to compare samples, with principal coordinate analysis (PCoA), using the plugin beta_diversity_through_plots.py and a sampling depth of 17236 (33). We applied the PERMANOVA method on the previously obtained dissimilarity matrices. PERMANOVA was performed using 1000 permutations. We computed alpha diversity metrics using non-phylogeny metrics (Observed OTUs, Chao1 index, Shannon index) with the plugin alpha_rarefaction.py and a sampling depth of 17236. We also performed Random Forest (RF) classification with 500 trees and ten-fold cross-validation using the plugin supervised_learning.py and the OTU table (34). To study longitudinal variation in our

samples, we used SplinectomeR, that applied smoothing splines to summarize data for straightforward hypothesis testing in longitudinal studies (35).

To compare samples collected in CRO and CTX mice, we used the LEfSe tool on the OTU table collapsed at genus level. LEfSe (Linear discriminant analysis Effect Size) determines the features (here, taxa collapsed at genus level) most likely to explain differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance (36). To identify significant associations between microbial and phenotypic variables, we applied a linear multivariate regression model specifically adapted to microbiome data: MaAsLin, Multivariate microbial Association by Linear models (37). MaAsLin constructs boosted, additive general linear models to associate metadata and transformed microbial taxonomic or functional relative abundances. Since microbial community profiles are typically high dimensional, boosting is used for feature selection over potential covariates to identify those most associated with each microbial feature. Selected metadata are then used in a general linear model with metadata as predictors and arcsin-square root transformed microbial relative abundances from the OTU table collapsed at genus level as the responses. The dataset generated and analyzed during the current study is available in the NCBI repository under the accession number PRJNA701545 (http://www.ncbi.nlm.nih.gov/bioproject/701545). The OTU table, collapsed at genus level is also provided as **Table S1**. Compositional biplot that simultaneously displays the sample clustering and phyla of the gut microbiomes of the fecal samples collected from CTX- and CRO-treated mice are represented in **Fig. S1**.

Pharmacokinetics

Stools collected were weighed, diluted in 0.9% NaCl (10 mg/100 µL) and homogenized by grinding in ball tubes and sonication. The ground material obtained was centrifuged (5 min, 13000 g, + 4 ° C).

For the CTX and desacetyl-CTX assay, 125 µL of the supernatant was mixed with 125 µL of a methanol/$ZnSO_4$ 3M solution (80/20 v/v) and 250 µL of an acetonitrile solution containing the deuterated internal standard ($^{13}C_2$, $^{2}H_3$-CTX). After centrifugation of the mixture (10 min, 13000 g, +4°C), 2 µL of the supernatant were injected into the chromatographic system (H-Class® Acquity UPLC system, Waters ™, St Quentin en Yvelines, France). The system consisted of a Kinetex 2.6 µm C18 column in a thermostatically controlled oven at 50°C, mobile phases with a binary gradient [(acetonitrile/formic acid 0.1% v/v) and (ultrapure water/formic acid 0.1% v/v)] at a flow rate of 0.8 mL/min and a mass spectrometer monitoring of the m/z ratios (456, 460, 414) for respectively CTX, $^{13}C_2$, $^{2}H_3$-CTX, and desacetyl-CTX for 4 min after each injection.

For the CRO assay, 250 µL of the supernatant was mixed with 250 µL of acetonitrile solution containing the deuterated internal standard (^{13}C, $^{2}H_3$-CRO). After centrifugation (5 min, 13000 g, + 4°C) of the mixture, 300 µL of the supernatant were mixed with 2 ml of dichloromethane. After centrifugation, 2 µL of the supernatant was injected into the H-Class® Acquity UPLC system. The chromatographic system consisted in a Kinetex 2.6 µm C18 column in a furnace thermostatically controlled at 50°C, mobile phases with a binary gradient (acetonitrile and a 1 mM aqueous ammonium acetate solution) at a flow rate of 0.8 mL/min and a mass spectrometer monitoring of the m/z ratios (555, 559) for the CRO and ^{13}C, $^{2}H_3$-CRO respectively for 4 min after each injection. For both methods, reproducibility and inaccuracy were less than 15%, the limit of quantification was 10 µg/g stool for CRO, CTX and desacetyl-CTX.

Quantification and statistical analysis

Statistical analyzes were performed using RStudio® (https://www.r-project.org/) and GraphPad Prism® 6.0 (GraphPad Software Inc. ™, La Jolla CA, USA). Areas under the curve (AUC) were calculated by trapezoidal method. Negative culture were fixed at 2 $\log_{10}$ CFU/g stool.

The Kruskal-Wallis test was used for unpaired data. The post hoc multiple comparison tests were performed using the Dunn method. Non-parametric Mann-Whitney tests were performed between the two-by-two groups with Bonferroni correction. All tests were defined with alpha risk determined a priori as significant if ≤ 0.05.

Results

CRO induced higher ESBL-*Kp* colonization than CTX

Results of growth culture are shown in **Figure 1A**. The absence of pre-existing 3GC-resistant strains prior to gavage by ESBL-producing *Kp* is demonstrated by the negativity of stool cultures on the day of gavage. CRO-induced ESBL-producing *Kp* colonization was consistently higher than CTX-induced colonization from day 4 to day 12 (p-value = 0.008, Mann-Whitney test) (**Figure 1B**).

CRO and CTX are both excreted in gut lumen

Detectable fecal concentrations for CRO were observed and were maintained until 10 days after the end of treatment (**Figure 1C**). Detectable concentrations for CTX and its active metabolite desacetyl-CTX were observed until 10 days after stopping treatment (**Figure 1D**). Fecal exposures to antibiotics were assessed using the area under the curve (AUC) of fecal

concentrations over time. AUC of CTX and desacetyl-CTX were 1015 $\mu g.g^{-1}$.day and 3962 $\mu g.g^{-1}$.day respectively. AUC of CRO was 1859 $\mu g.g^{-1}$.day.

CRO and CTX drive the architecture of the gut microbiota in different trajectories

At baseline (i.e., before antibiotic treatment was started), we did not find any significant difference between CTX, CRO-treated mice and control mice, in terms of alpha diversity (ANOVA, Shannon index: p-value= 0.556, observed OTUs: p-value = 0.143). Overall, we found a significant decrease in alpha diversity between antibiotic-treated mice and control mice (ANOVA, Shannon index: p-value = 0.001, observed OTUs: p-value = 0.001, chao1 index: p-value = 0.001). However, we did not find significant difference in alpha diversity between CTX and CRO-treated mice (ANOVA, Shannon index: p-value = 0.665, observed OTUs: p-value = 0.530, chao1 index: p-value = 0.855, **Fig. S2**). Importantly, alpha diversity decreased significantly with time in both CTX and CRO-treated mice (p-value = 0.01), but this decrease was not different between CTX-treated mice and CRO-treated mice (p-value = 0.50).

Using Unweighted UniFrac distances, at baseline (i.e., before antibiotic treatment was started), we did not find any significant difference between we did not find any significant difference between CTX, CRO-treated mice and control mice (PERMANOVA, $r^2 = 0.068$, p-value = 0.492, **Figure S3A**). Overall, we found that both CTX and CRO significantly alter the overall architecture of the gut microbiota when compared to control mice (PERMANOVA, $r^2 = 0.128$, p-value = 0.001, principal component 1: ANOVA, p-value < 0.001, principal component 2: ANOVA, p-value < 0.001). Moreover, CRO and CTX drove the architecture of the gut microbiota in 2 different trajectories (PERMANOVA, $r^2 = 0.142$, p-value = 0.001,

283 principal component 1: ANOVA, p-value = 0.358, principal component 2: ANOVA, p-value
284 = 0.002, **Figure S3A**).

285 We observed that the time after the antibiotic treatment had a strong impact on the
286 beta-diversity with higher Principal Coordinate (PC) 1 and PC2 scores observed at day 12
287 (**Fig. 2A**). Before treatment and until day 4, CRO- and CTX-treated mice were similar and
288 differences appeared at day 8 and increased at day 12. A more important inter-individual
289 variability was observed in CTX-treated than in CRO-treated mice at day 12. Beta-diversity
290 trajectories by mouse are represented in **Fig. S3B**. Supervised learning using Random Forests,
291 a machine learning method using OTUs as predictive features, accurately assigned samples to
292 their source population (CTX or CRO-treated mice) based on taxonomic profiles at the OTU
293 level (83.3% accuracy, 3 times better than the baseline error rate for random guessing). Thus,
294 based on microbiome data only, we were able to predict if a mouse received CRO or CTX
295 treatment.

296 Using LEfSe on the OTU table collapsed at the genus level (36), we found that 12 taxa were
297 significantly different between CTX and CRO-treated mice. Specifically, CRO-treated mice
298 were associated with a significant gain in *Lactobacillus*, *Klebsiella*, unclassified
299 *Enterobacteriaceae*, and *Parabacteroides* when compared to CTX-treated mice, that were,
300 conversely, associated with increase in *Enterococcus*, unclassified *Carnobacteriaceae*,
301 unclassified *Planococcaceae*, *Granulicatella*, unclassified *Lactobacillales*, unclassified
302 *Enterococcaceae*, *Vagococcus* and *Anaeoplasma* (**Figure 2B**). The most significant
303 differentiating taxa between CTX and CRO-treated mice are represented in **Fig. S4A and B**.
304 Significantly different taxa between CRO and control mice and CTX and control mice
305 respectively are described in **Fig. S4C** and **5D**. Comparison of antibiotic treated mice (all
306 collected samples in CTX and CRO-treated mice) and control mice (all collected samples) is
307 represented un **Table S3**.

To confirm these changes, we used SplinectomeR that enables group comparisons in longitudinal microbiome studies. First, we plotted the longitudinal changes in the three groups of mice, antibiotic-treated mice (CRO or CTX) and control mice. We found that in control mice, gut microbiota composition was relatively stable along time, based on the OTU table collapsed at genus level (**Fig. S5A**), whereas, gut microbiota composition was dramatically altered following CRO and CTX treatment (**Fig. S5B and C**). We confirm that *Enterococcus* was significantly higher in CTX than in CRO-treated mice along days of collection (p-value=0.01, **Figure 2C**) and that *Klebsiella* was significantly higher in CRO than in CTX-treated mice (p-value = 0.01, **Figure 2D**). Unclassified *Enterobacteriaceae*, *Parabacteroides* and *Lactobacillus* were also higher in CRO than in CTX-treated mice (p-value = 0.04, p-value = 0.03 and p-value = 0.1, respectively).

Antibiotic fecal concentrations impact on OTU abundance

We investigated the relationship between CRO, CTX and desacetyl-CTX fecal concentrations and the gut microbiome by identifying significant multivariate linear associations using MaAsLin. The relative abundance of 34 OTUs was positively correlated with CRO fecal concentration levels, including members of *Sphingomonadaceae*, *Microbacteriaceae*, *Sphingobacteriaceae* and *Enterobacteriaceae* families. Two OTUs were positively correlated with CTX/desacetyl-CTX fecal concentration levels, corresponding to *Lactobacillus* and *Leucobacter* genera.

We observed that higher antibiotic concentration levels were not associated with highest PC1 and PC2 scores but preceded these metagenomics alterations leading to colonization by ESBL-producing *Kp*.

ESBL-producing *Klebsiella pneumoniae* carriage is associated to taxonomic changes

We observed that clustering of fecal samples was partly driven by the increased carriage of ESBL-producing *Kp* (r^2 = 0.0641, p-value = 0.001, **Fig. S6**). Higher colonization levels being associated with higher PC1 and PC2 scores in the two groups, i.e. with the most altered microbiota. This beta-diversity pattern was mainly found in CRO-treated mice and especially at day 12 as described in **Fig. S3B** and suggested that CRO selects a microbial community which promotes colonization by ESBL-producing *Kp*. Conversely, highest inter-individual diversity observed at day 12 in CTX-treated mice was associated with in a lower level of colonization. We found that 8 taxa from the OTU table collapsed at genus level, were significantly different between mice displaying a high level of ESBL-producing *Kp* and those displaying a low level (**Fig. S6B**). Specifically, mice with a high level were associated with a significant gain in *Klebsiella* and unclassified *Pseudomonadaceae* when compared to low level mice, that were, conversely, associated with increase in unclassified *Bacteroidales*, unclassified *Desulfovibrionaceae*, *Sutterella*, unclassified *Peptococcaceae*, unclassified *Clostridiaceae* and *Akkermansia*.

These results were confirmed by a negative association found between intensity of ESBL-producing *Kp* carriage and OTUs from *Bacteroidales* and *Clostridiaceae* (**Table S2**).

Discussion

This experimental study shows three main results: (i) CRO appears to promote higher fecal carriage of ESBL-producing *Kp* than CTX; (ii) both antibacterial agents altered fecal microbiotas, but CRO had a more important impact than CTX; (iii) higher level of fecal colonization by ESBL-producing *Kp* was associated with more altered microbiota. These results suggest that the effect of CRO on *Kp* colonization could be mediated by its effect on the intestinal microbiota.

We observe that fecal *Kp* levels were significantly higher in CRO-treated mice than in 2 other groups (CTX and NaCl) but were equivalent in control and CTX-treated mice. We cannot explain these results neither by the fecal concentrations observed during the experience nor by the MIC of the strain. Indeed, concentrations of CRO and CTX in the feces were they similar to those observed by previously published studies (17,25,26). Moreover, we cannot stated that MIC of CRO and CTX were different regarding the variability in measurement (38). Therefore, we made 2 hypotheses: the first based on the modification of the architecture of the microbiota and the second based on the mechanism of action of these 2 3GC.

Indeed, we found that CRO and CTX drove the architecture of the gut microbiota in 2 different trajectories. As expected, we found a significant decrease in alpha diversity between antibiotic-treated mice and control mice but we did not find significant difference in alpha diversity between CTX and CRO-treated mice (16). If before the treatment and until day 4, it was impossible to tell the difference between CRO- and CTX-treated mice, differences appeared at day 8 and were accentuated at day 12. A more important inter-individual variability was observed in CTX-treated mice which do not reached all the maximum values of PC1 and PC2 at day 12 contrary to CRO-treated mice which reached high PC1 and PC2 values. Interestingly, higher *Kp*-colonization levels were associated with more altered microbiota in the two groups and higher antibiotic concentration levels were not associated with highest PC1 and PC2 scores but were preceded by these metagenomics alterations. Conversely, highest inter-individual diversity observed at day 12 in CTX-treated mice was associated with a lower level of colonization. These data suggested that CRO could select a microbial community which promotes colonization by ESBL-producing *Kp* more easily than CTX. These modifications have not been observed by Burdet *et al*. but their work focused on low dosages of CRO given over only 3 days and in healthy individuals which are not comparable to those potentially given in more serious infections (16). The only 2 subjects

with changes in the microbiota were those with detectable CRO in the stool but previous study that CRO is more frequently found in the stool in high concentrations and similar to that which we find (16,26).

Our results challenge also the pharmacokinetic hypothesis considering that CRO only could selected more ESBL-producing *Enterobacteriaceae* because of a higher fecal elimination than CTX (12). As explain before, we observed detectable concentration of CRO, CTX and its active metabolite desacetyl-CTX until 10 days after stopping treatment in faeces (39). These results confirm those of Grall *et al.* who found digestive excretion of CTX in significant proportions (17). We could also hypothesize that desacetyl-CTX plays a role in the prevention of carriage ESBL-producing *Enterobacteriaceae*. Indeed, if the bactericidal activity of desacetyl-CTX is generally considered to be lower than CTX (the MIC of desacetyl-CTX is 8-fold higher than that of CTX on susceptible strains of *Kp*), desacetyl-CTX could act as a beta-lactamase inhibitor and could potentiate the action of CTX as suggested by previous studies (40,41). To reinforce this hypothesis, Labia *et al* showed that desacetyl-CTX was much more stable to hydrolysis by beta-lactamase than CTX and could have a sustained bactericidal effect even in the presence of these enzymes (42).

These data are crucial for the comprehension of the parameters influencing the carriage of ESBL-producing *Enterobacteriaceae*, especially for patient displaying a high risk of colonization such as patient treated with antibiotics or travelers in endemic area.

Our study has several limitations. First, we used 16S rRNA sequencing that limits taxonomic identification at genus level. Therefore, we were not able to identify species or strains that differ between CRO and CTX treated. In addition, our work is limited to a single strain of ESBL-producing *Enterobacteriaceae* and deserves to be strengthened by testing other strains.

Conclusions

In mice, both CRO and CTX modify the microbiota but CRO promotes more the carriage of a strain of ESBL-producing *Kp*. If CRO appears to select a favorable specific microbial community for the installation of this bacteria in a concentration-dependent way, CTX and particularly its active metabolite desacetyl-CTX could prevent colonization because of a beta-lactamase inhibitor effect.

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Authors' contributions

M.G., E.M. and E.D. designed the experiments, M.G., F.B., R.B. conducted animal experiments, M.G., F.B., R.B., F.J. and P.B. conducted bacterial culture, MG., P.A., A.B. and J.G. conducted functional exploration experiments, Q.L. and E.M. performed metagenomics analysis, F.B., R.B. and E.D. conducted pharmacokinetics measurements, M.G., F.B., E.M, E.D., E.B, D.L. and M.N. wrote the manuscript and created figures.

Ethics approval and consent to participate

Ethics approval was not required for the study.

Competing interests

The authors declare no competing interests.

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Figure

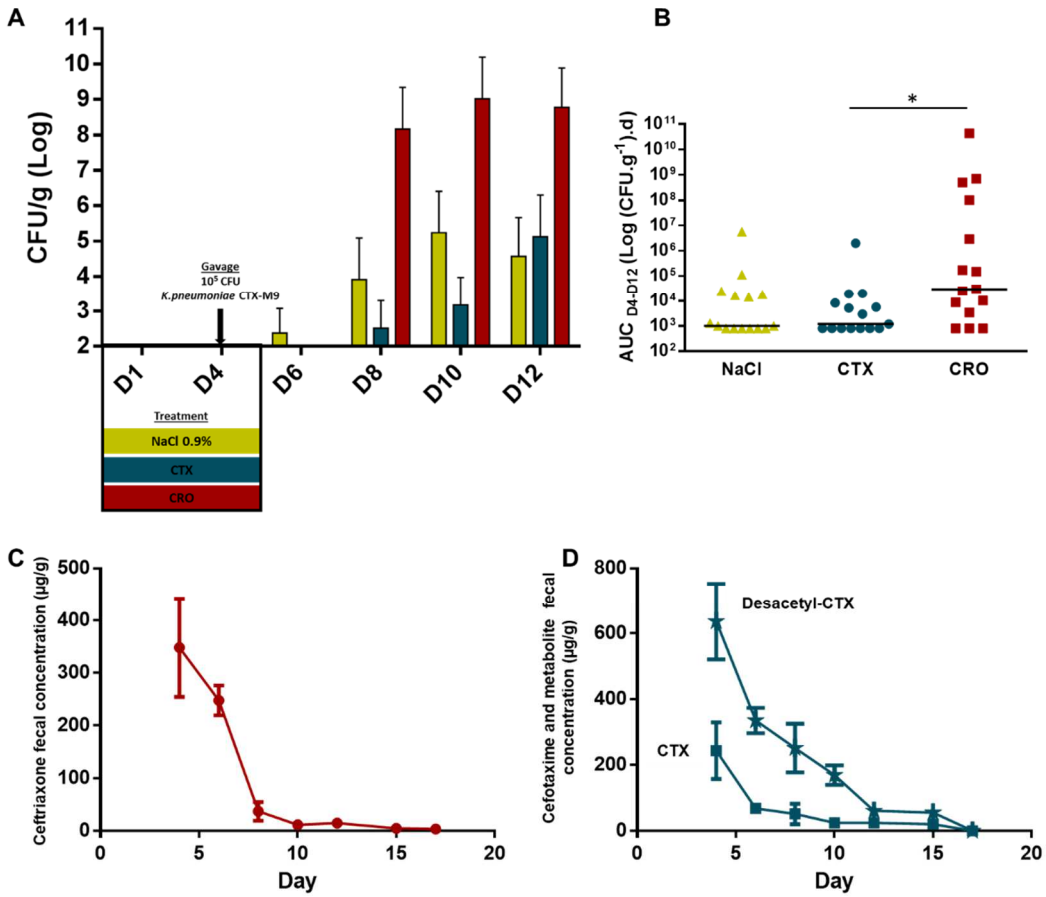
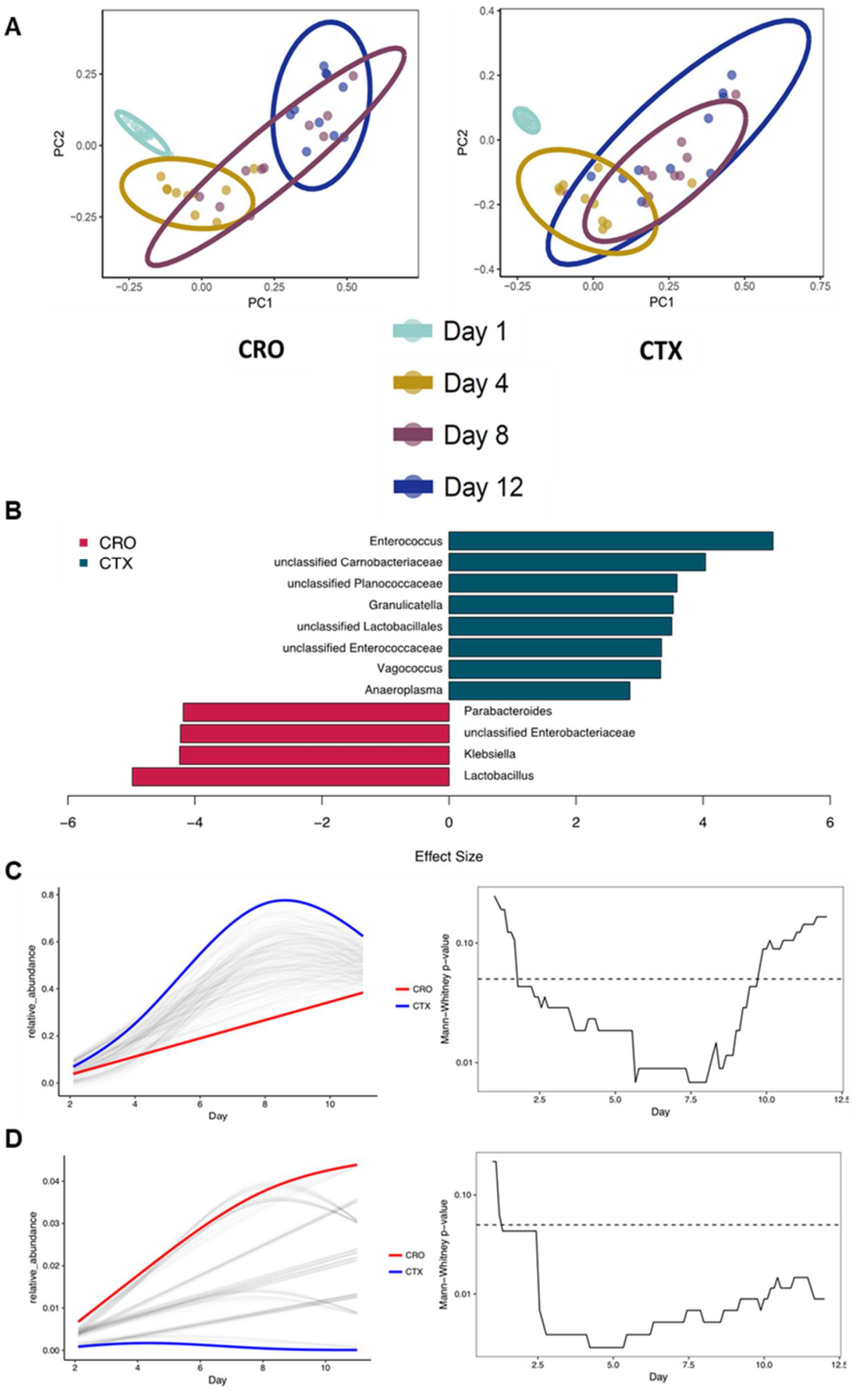


Figure 1: Gut colonization by *Klebsiella pneumoniae*, transit modifications and fecal concentrations of CRO and CTX

(A) Quantification of fecal excretion of ESBL-producing *Kp* over time according to treatment administered (n = 15 per group). The bars represent the mean and the range. (B) Quantification of fecal excretion of ESBL-producing *Kp* over time according to treatment administered (n = 15 per group). Lines correspond to loess-smoothed conditional means and shading to SE. (C) Area under the curve (AUC) of ESBL-producing *Kp* excretion of control, CTX and CRO groups. A statistically significant difference in colonization between CRO and CTX was observed from day 4 to day 12 (p-value = 0.0078, Mann-Whitney test) and is

symbolized by an asterisk. (D) CRO fecal concentrations. (E) CTX (squares) and desacetyl-CTX (stars) fecal concentrations. Values were indicated as mean +/- SEM (n = 10 per group). Detection threshold was 10 μg/g.


A
PC2
PC1
CRO
CTX
Day 1
Day 4
Day 8
Day 12
B
CRO
CTX
Enterococcus
unclassified Carnobacteriaceae
unclassified Planococcaceae
Granulicatella
unclassified Lactobacillales
unclassified Enterococcaceae
Vagococcus
Anaeroplasma
Parabacteroides
unclassified Enterobacteriaceae
Klebsiella
Lactobacillus
Effect Size
C
relative_abundance
Day
Mann–Whitney p-value
D

582

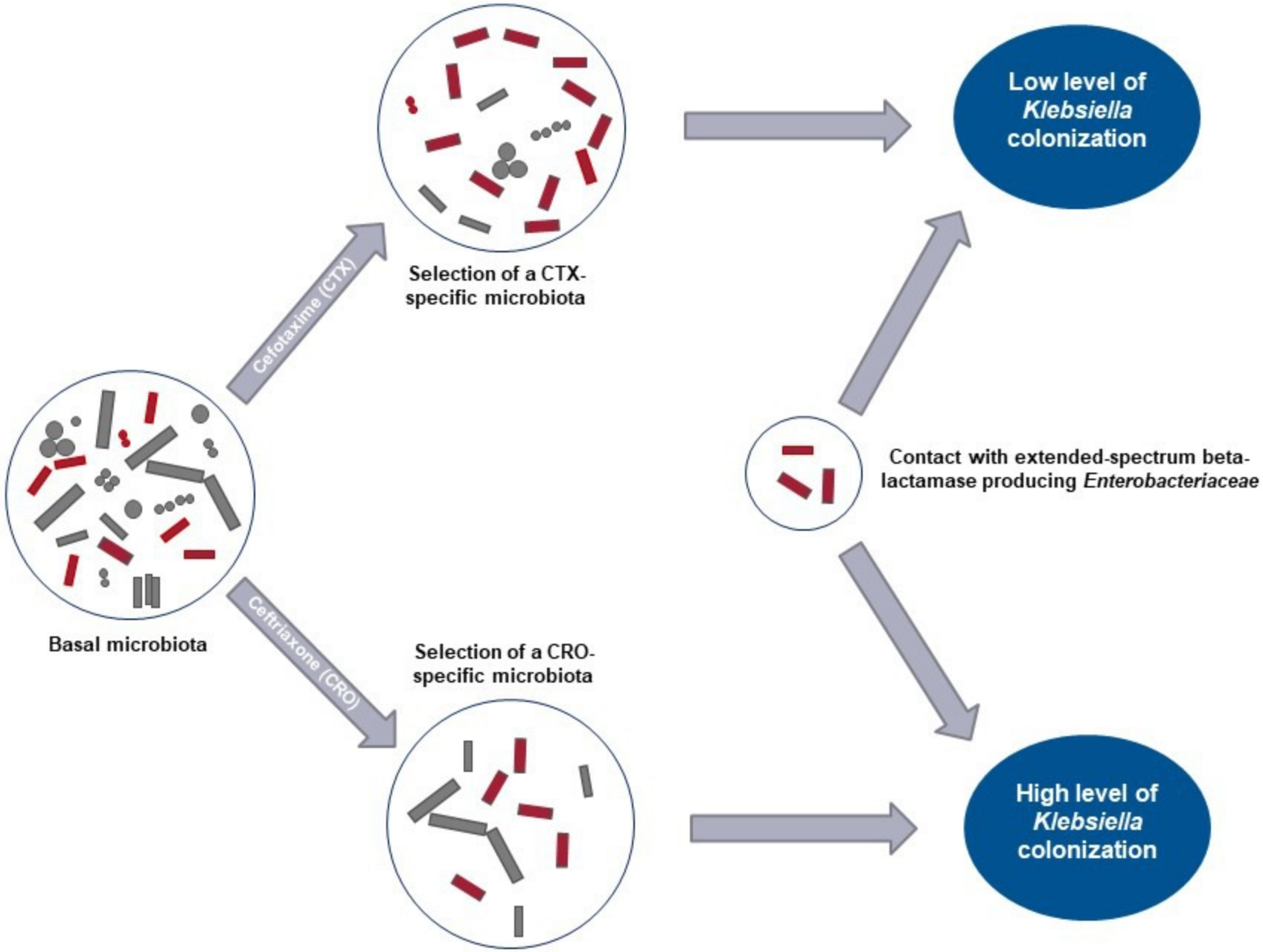
Figure 2: CRO and CTX alter the overall architecture of gut microbiota and induce taxonomic changes

(A) Beta diversity comparisons of the gut microbiomes of the fecal samples collected from CTX- and CRO-treated mice. Beta diversity is represented by ellipse clustering according to the day after the start of antibiotic treatment.

(B) Summary of the taxa that differentiate CTX from CRO-treated mice using Linear discriminant analysis Effect Size analysis (LEfSe). At genus level, 12 taxa were significantly different CTX from CRO-treated mice (absolute LDA log 10 score >2).

(C) Longitudinal changes in *Enterococcus* compared between CTX and CRO-treated mice, using SplinectomeR with permuted spline test. (Left) *Enterococcus* relative abundance over time distinguishes CTX (group spline in blue) and CRO-treated mice (group spline in red; 999 permutations, p-value = 0.01). Permuted splines represented in grey. The permuted splines lie predominantly between the two observed curves, supporting the conclusion that this difference is larger than expected by chance. (Right) The plot output of the sliding spliner function shows the p-value at each specified interval derived from the distribution of points from individuals' smoothed splines Dotted line indicates p-value = 0.05.

(D) Longitudinal changes in *Klebsiella* compared between CTX and CRO-treated mice, using SplinectomeR with permuted spline test. (Left) *Klebsiella* relative abundance over time distinguishes CTX (group spline in blue) and CRO-treated mice (group spline in red; 999 permutations, p-value = 0.01). Permuted splines represented in grey. The permuted splines lie predominantly between the two observed curves, supporting the conclusion that this difference is larger than expected by chance (Right) The plot output of the sliding spliner function shows the p-value at each specified interval derived from the distribution of points from individuals' smoothed splines. Dotted line indicates p-value = 0.05.


Low level of *Klebsiella* colonization
Selection of a CTX-specific microbiota
Cefotaxime (CTX)
Basal microbiota
Ceftriaxone (CRO)
Selection of a CRO-specific microbiota
Contact with extended-spectrum beta-lactamase producing *Enterobacteriaceae*
High level of *Klebsiella* colonization