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Title

Ceftriaxone and cefotaxime have similar effects on the intestinal microbiota in human volunteers treated by standard doses regimens

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Running title

Ceftriaxone vs cefotaxime on microbiota

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Abstract

Background

Ceftriaxone has a higher biliary elimination than cefotaxime (40% vs 10%), which may result in a more pronounced impact on the intestinal microbiota.

Methods

We performed a monocenter, randomized open-labelled clinical trial in 22 healthy volunteers treated by intravenous ceftriaxone (1g/24hrs) or cefotaxime (1g/8hrs) for 3 days (ClinicalTrials.gov NCT02659033). We collected fecal samples for phenotypic analyses, 16S rRNA gene profiling and measurement of antibiotic concentration, and compared between groups the evolution of microbial counts and indices of bacterial diversity over time. Plasma samples were drawn at day 3 for pharmacokinetic analysis.

Results

Emergence of 3rd generation cephalosporin resistant Gram-negative enteric bacilli (*Enterobacterales*), *Enterococcus* spp., or noncommensal microorganisms were not significantly different between groups. Both antibiotics reduced the counts of total Gram-negative enteric bacilli and decreased bacterial diversity, without significant difference between groups. All but one volunteer from each group exhibited undetectable levels of antibiotic in feces. Plasma pharmacokinetic endpoints were not correlated to alteration of bacterial diversity of the gut.

Conclusions

Both antibiotics markedly impact the intestinal microbiota, without any significant difference when standard clinical doses were administered for 3 days. This might be related to similar daily amounts of antibiotics excreted through the bile using a clinical regimen.

Background

The rise in bacterial resistance to antibiotics, particularly that of Gram-negative bacteria, constitutes a serious threat to our medical system (1). Initially restrained to nosocomial infections, antibiotic resistance has spread worldwide (2), affecting treatment of community-acquired infections. One of the drivers of this evolution is the impact that antibiotics, in particular β -lactams, exert on the intestinal commensal microbiota of both humans and animals (3).

In their studies of healthy volunteers receiving 5-day courses of ciprofloxacin, Dethlefsen *et al.* observed that antibiotic administration had a rapid effect in reducing bacterial diversity and in modifying the gut microbiome composition (4, 5). Similar observations were reported with moxifloxacin (6). These results are a strong incentive to better document the impact of antibiotics on the gut microbiome (7), and to identify the drivers of its disruption. Indeed, the gut microbiome has been shown to contribute to health maintenance of its host (8).

A limited number of studies suggested that taking into account antibiotics pharmacokinetic characteristics might help to reduce their impact on the microbiome. The two 3rd generation cephalosporins ceftriaxone and cefotaxime share their antibacterial spectrum, indications, and intravenous administration route, but have different pharmacokinetics characteristics: ceftriaxone is administered once daily and has a 40% biliary elimination, while cefotaxime needs thrice daily administrations but has a 10% biliary elimination. In two randomized controlled trials, female patients requiring gynaecological surgery received a single high dose (2 grams of ceftriaxone or 2 grams of cefotaxime) antibiotic prophylactic treatment (9, 10). Both studies suggested a more pronounced impact of ceftriaxone on the gut microbiota in terms of selection of Gram-negative enteric bacilli populations resistant to 3rd generation cephalosporins. Similar trends were reported in observational studies (11, 12).

However, no study dealing with usual antibiotic regimens administered in clinical practice is available. Indeed, cefotaxime is usually administered at higher daily doses than ceftriaxone, which may ultimately lead to quite similar effects on the gut microbiota. Moreover, next generation sequencing methods were not used to investigate this question but may provide a precise description of the microbiome.

Here, we report the results of a prospective randomized clinical trial performed in healthy volunteers receiving a clinical course of ceftriaxone or cefotaxime for 3 days for the comparison of their impact on the gut microbiota using phenotypic analysis and 16S rRNA gene profiling.

Methods

Study design

We conducted a prospective, open label, randomized clinical trial from March 2016 to August 2017 in adult healthy volunteers in the Clinical Investigation Center at Bichat – Claude Bernard Hospital (Paris, France). All participants received oral and written information, and provided signed consent before inclusion. The trial obtained approval from the Independent Ethics Committee “Île-de-France 1” on 12/21/2015 (2015-oct-14028) and from the National Agency for Security of Medicinal Products on 07/24/2015 (150527A-41), and was conducted in respect with Good Clinical Practice and the Declaration of Helsinki as last amended. It was sponsored by Assistance Publique – Hôpitaux de Paris.

Subjects selection criteria

Male and female healthy volunteers between 18 and 65 years were eligible if their body mass index was between 18.5 and 30 kg/m², if they had normal digestive transit and if they were considered healthy by medical history, vital signs, physical examination, electrocardiogram and blood laboratory results at a screening visit 16-26 days prior to randomization. Females in child-bearing age were eligible if they had a contraceptive treatment and a negative pregnancy test at screening.

Subjects with a history of hospitalization within the last 6 months or antibiotic exposure within the past 3 months were not eligible, neither were those with any history of chronic active disease including HIV, HCV or HBV infection, a history of allergy to β -lactams or who were under legal protection. Subjects' household could neither have any active chronic disease nor have received any antibiotic treatment in the preceeding 15 days. They were secondary excluded if they did not provide any fecal sample before randomization, or if more than 1 fecal sample was missing between day 1 and day 7 after antibiotic treatment initiation.

Treatments

Volunteers were randomised (1:1 ratio) to receive from day 1 to day 3, either ceftriaxone (1g/24 hours) or cefotaxime (1g/8 hours) as 30-minute intravenous infusions using an automatic high-precision infusion pump. Day 1 was defined as the first day of antibiotic treatment.

Plasma sampling and analyses

For each volunteer, six blood samples were collected at day 3 for determination of total concentration of antibiotics in plasma. In the ceftriaxone treatment group, blood sample were collected just before and at 0.5, 1, 2, 4 and 8 hours after the beginning of the 3rd infusion. In the cefotaxime treatment group, blood samples were collected just before and at 0.5, 1, 2, 4 and 6 hours after the 7th infusion. Exact times of beginning and end of infusion were recorded, as well as exact sampling times. Blood samples were taken from the arm opposite that of antibiotic administration.

Blood samples were immediately centrifuged (4000 rpm for 5 minutes) and plasma was stored at -80°C until analysis. Total plasma concentration of ceftriaxone and cefotaxime were determined by

HPLC coupled with ultraviolet spectrophotometric detection (280 nm for ceftriaxone and 254 nm for cefotaxime). The limit of quantification was 0.5 mg/L for both antibiotics.

Fecal sampling and analyses

A total of 13 fecal samples was obtained from each participant (at days -15±2, -6±2, -1, 1, 2, 3, 4, 7, 10±1, 15±1, 30±3, 90±7 and 180±7). All bacteriological analyses were performed following the PROBE guidelines (13).

Determination of bacterial counts in feces

Fecal samples were stored at 4°C after emission and transmitted to the bacteriology laboratory after blinding. One hundred mg of feces were suspended in 1 mL of brain–heart infusion broth containing 30% glycerol and stored at -80°C.

Total Gram-negative enteric bacilli (*Enterobacterales*) were counted by plating serial dilutions of broth on Drigalski agar (bioMérieux, Marcy-l’Etoile, France). Third generation resistant Gram-negative enteric bacilli were detected and counted on ChromID® ESBL agar (bioMérieux, Marcy-l’Etoile, France) and biplate ESBL agar (AES Chemunex, Ivry-sur-Seine, France) (37°C for 48h). All distinct colonies were studied. *Enterococcus* spp. were detected and counted using Enterococcosel plates (Becton, Dickinson and Company, USA). Intestinal colonization by yeasts, *Pseudomonas aeruginosa*, *Clostridium difficile* and *Staphylococcus aureus* was detected and counted on ChromID® Candida (bioMérieux), Cetrimide agar (Oxoid, Basingstoke, UK), ChromID® *Clostridium difficile* (bioMérieux) and BBL™ Mannitol Salt Agar (Becton Dickinson and Company, Franklin Lakes, New Jersey, USA), respectively. The limit of quantification was 2.0 log₁₀ CFU/g of feces for all microorganisms.

Third generation resistant Gram-negative enteric bacilli strains were tested for antibiotic susceptibility by the disk diffusion method according to EUCAST. The presence of ESBL was detected using the double-disk synergy test. Overproduction of intrinsic or plasmid cephalosporinase AmpC was detected by studying susceptibility on cloxacillin agar (14). Resistant strains were identified by mass spectrometry (Maldi Biotyper, Bruker Daltonics, Bremen, Germany). Third-generation cephalosporin-resistant *E. coli* strains were PCR-based typed or sequenced (Illumina technology) for determination of the molecular support of resistance and the phylogroup, sequence type and O:H type determination (see Supplementary Text S1). Sequence data have been submitted to the EBI database under accession number PRJEB28341.

16S rRNA gene profiling of the intestinal microbiota

Samples obtained at days -15±2, -1, 4, 7, 10±1, and 30±3 were also analysed by 16S rRNA gene profiling. Operational Taxonomic Units (OTUs) were aggregated at the phylum level and relative abundance of each bacterial phylum was determined. Various indices of diversity were computed. α-diversity (intra-sample) metrics included the Shannon diversity index (15) and the number of observed OTUs. β-diversity (inter-sample) metrics included the Bray-Curtis dissimilarity (16) and the unweighted

UniFrac distance (17). Detailed methods are presented in Supplementary Text S1. Sequence data have been submitted to the EBI database under accession number PRJEB28341.

Determination of fecal antibiotic concentration

Fecal samples were stored at -80°C until the assay was performed. Active fecal concentrations of ceftriaxone and cefotaxime (and their metabolites) were measured on samples collected between day -1 and day 10 by microbiological assay (with *E. coli* ATCC25922 for both antibiotics) after incubation at 37°C for 24 hours, with a limit of quantification of $1.25\ \mu\text{g/g}$ for both antibiotics.

Pharmacokinetic and bacteriological endpoints used for treatments comparison

The primary endpoint was the area under the curve between baseline (defined as day 0) and day 7 (AUC_{D0D7}) of the log₁₀-counts of 3rd generation cephalosporins resistant Gram-negative enteric bacilli.

Bacteriologic secondary endpoints included the followings: $\text{AUC}_{\text{D0D15}}$ of the variation from baseline of the log₁₀-counts of 3rd generation cephalosporins resistant Gram-negative enteric bacilli, AUC_{D0D7} and $\text{AUC}_{\text{D0D15}}$ of the variation from baseline of the log₁₀-counts of total Gram-negative enteric bacilli, of *Enterococcus* spp. and of noncommensal microorganisms of the intestinal microbiota and proportion of uncolonized subjects with the emergence of 3rd generation cephalosporins resistant Gram-negative enteric bacilli or noncommensal microorganisms in the intestinal microbiota between day 1 and day 15.

The pharmacokinetic endpoints were the area under the curve of the free plasma concentration over time over 24 hours ($f\text{AUC}_{0-24,ss}$), the maximal and trough free plasma concentrations (fC_{max} and fC_{min} , respectively) at steady state, and the fraction of time during which the free plasma concentration is above 1 mg/L at steady state ($fT>1$) (18).

As an exploratory analysis, we computed for each subject the $\text{AUC}_{\text{D-1D30}}$ of the variation from day -1 to day 30 of the 2 indices of α -diversity. For β -diversity indices, for each sample obtained at day x, we determined the index value for each subject between day x and day -1 and computed the $\text{AUC}_{\text{D-1D30}}$ of the diversity index.

Statistical methods

The sample size of the trial was computed using data from the study by Michea-Hamzehpour et al. (9). Assuming a common standard deviation of 2.2 log₁₀ CFU/gram of stool, the inclusion of 12 subjects in each group was required to support a 3 log₁₀ CFU/g difference between treatment groups, with a 90% power and a type I error of 0.05. Due to recruitment difficulties, inclusions were stopped on 04/18/2017. At this date, 22 volunteers had been recruited (11 in each group). With this number, the power of the trial was estimated at 86% using the above hypotheses.

We computed the variation from baseline of the bacterial and fungal counts in feces, and computed the AUCs using actual date and time of stools emission and the trapezoidal method. For each subject,

the baseline value was computed as the arithmetical mean of the log₁₀ counts observed on available pre-treatment samples. The AUC_{D0D15} (respectively AUC_{D-1D30}) was normalized using the observed delay between day 0 (resp day -1) and the actual time of collection of the day 15 (respectively day 30) sample.

Plasma concentrations of antibiotics were analysed using the population approach (see Supplementary Text S1). Pharmacokinetic endpoints were derived for each antibiotic at steady state, assuming 90% protein binding for ceftriaxone (19) and 40% for cefotaxime (20). Derived pharmacokinetic endpoints ($fAUC_{0-24,ss}$, fC_{max} , fC_{min} and $fT>1$ mg/L) were computed for each subject using the predicted individual pharmacokinetic profiles. We used the 1 mg/L threshold as this value corresponds to the susceptibility breakpoint of *Enterobacteriaceae* species for ceftriaxone and céfotaxime (http://www.eucast.org/clinical_breakpoints/).

Endpoints were compared between groups using bilateral non parametric Wilcoxon or Fisher exact tests as appropriate, and a type I error of 0.05. We computed 95% confidence intervals of proportions using the binomial distribution. We analyzed the link between each pharmacokinetic endpoint and the AUC_{D-1D30} of studied diversity indices using the Spearman rank correlation coefficient.

Data are presented as median (min; max) or n (%). Statistical analyses were performed using the SAS 9.4 software (SAS Institute, USA).

Results

Subjects

Thirty-three subjects were screened and 22 subjects were randomized (11 in each treatment group); all participated in the trial until the end of follow up. Their baseline characteristics are presented in Table 1. No serious adverse event was reported, but treatment was stopped early in one subject of the cefotaxime group who presented a vasovagal malaise while placing the intravenous line for the 8th infusion.

Ceftriaxone and cefotaxime pharmacokinetics

For both antibiotics, plasma pharmacokinetics were best described by a 2-compartment model with first order elimination from the central compartment. All population parameters could be estimated with reasonable precision (Supplementary Table S1), and goodness of fit was satisfactory (Supplementary Figures S1 and S2). Derived pharmacokinetic endpoints estimated for the 11 included subjects of each treatment group are presented in the Table 2.

All subjects exhibited undetectable fecal concentrations of antibiotics in feces between day -1 and day 30, except one in each treatment group. Subject 16 had detectable concentrations of ceftriaxone between day 2 and day 7. Concentrations ranged between 5.0 µg/g and 93.7 µg/g, the maximal value being measured at day 4. Subject 3 had detectable fecal concentrations of cefotaxime in feces, at day 4

(1.6 µg/g). These 2 subjects had derived pharmacokinetic endpoints close to the median values observed in their respective treatment groups.

Phenotypic analysis of the fecal samples

Overall, 283 fecal samples were available for phenotypic analyses. Baseline counts of studied microorganisms are presented in Table 1.

3rd generation cephalosporins resistant Gram-negative enteric bacilli

No major change in the counts of resistant Gram-negative enteric bacilli was observed in either of the two treatment groups over time (Figure 1 and Supplementary Figure S3, panels A & B), and no significant difference was observed between groups (Table 3). Molecular characteristics of detected resistant strains are presented in Supplementary Table S2.

At baseline, colonization with resistant Gram-negative enteric bacilli was detected in 5 subjects (45.5%) and 1 subject (9.7%) in the ceftriaxone and cefotaxime groups, respectively. Among uncolonized subjects, 1 (16.7%, 95%CI: 0.4%; 64.1%) from the ceftriaxone group and 2 (20%, 95%CI: 2.5%; 55.6%) from the cefotaxime group acquired resistant Gram-negative enteric bacilli between the beginning of treatment and day 15 ($p>0.99$, Table 4).

Other studied microorganisms

No significant difference between groups was observed regarding the variation from baseline of any other studied microorganisms (Table 3, Figure 1 and Supplementary Figure S3, panels C-H). Both antibiotics profoundly reduced the counts of total Gram-negative enteric bacilli, with a highest median reduction from baseline of 4.4 log₁₀ CFU/g (2.4; 8.7) in the ceftriaxone group and 3.8 (1.9; 8.1) in the cefotaxime group (Figure 1 and Supplementary Figure S3, panel C). At day 15, counts of Gram-negative enteric bacilli returned to their baseline value. Carriage of toxigenic *C. difficile* was detected in 3 patients, 1 (at day 10) in the ceftriaxone group and 2 (1 at day 4 and 1 at day 15) in the cefotaxime group ($p>0.99$). Results regarding the emergence of other noncommensal microorganisms of the intestinal tract are presented in Table 4.

16S rRNA gene profiling of the fecal samples

16S rRNA gene profiling was performed on 148 available samples. Baseline characteristics of taxonomic composition and diversity indices are presented in Table 1.

Taxonomic composition

Both antibiotics reduced the relative abundance of *Firmicutes* (median maximal change of -9.3% (-47.6; 2.2) in the ceftriaxone group and -12.3% (-22.4; -4.0) in the cefotaxime group), of *Actinobacteria*, (-3.6% (-11.0; 0.0) in the ceftriaxone group and -2.3% (-8.9; -0.1) in the cefotaxime group) and of *Bacteroidetes* (-2.2% (-31.4; 10.8) in the ceftriaxone group and -4.0% (-23.8; 15.2) in the cefotaxime group). The relative abundance of *Proteobacteria* remained roughly unchanged (median maximal

change of -0.5% (-1.6; 0.0) in the ceftriaxone group and 0.0% (-5.7; 0.2) in the cefotaxime group) (Figure 2).

Bacterial diversity

Although no significant difference was observed between ceftriaxone and cefotaxime (Table 3), both antibiotics exhibited a profound impact on bacterial diversity. The highest reduction of the Shannon index and number of OTUs from baseline was 0.8 Shannon unit [0.3; 2.9] and 100 OTUs [40; 268] in the ceftriaxone group, and 1.1 Shannon unit [0.2; 1.8] and 144 OTUs [97; 234] in the cefotaxime group (Figure 3 and Supplementary Figure S4).

At day -1, interindividual UniFrac distances appeared quite homogeneous except for subject 3 (from the cefotaxime group), whose distance from other subjects was notably higher (Supplementary Figure S5). His distance to the others increased at day 4, whereas other interindividual distances remained roughly unchanged. At day 7, subject 3 and subject 16 (from the ceftriaxone group) were very distant from the other subjects. These 2 subjects are those who exhibited detectable concentrations of antibiotics in feces. They also exhibited the highest changes of diversity over time (Figure 3). Similar patterns were observed for the Bray-Curtis dissimilarity (Supplementary Figure S6).

At day 30, α -diversity indices returned to their baseline value, while β -diversity indices between day -1 and day 30 were similar to values observed between day -15 and day -1 in both groups (Figure 3 & Supplementary Figure S7), attesting of the temporal intraindividual variability of the microbiome composition.

Pharmacokinetic endpoints were not significantly correlated with any index of bacterial diversity (Supplementary Table S3).

Discussion

Preclinical and clinical data reported differences in ceftriaxone and cefotaxime pharmacokinetic characteristics, in particular regarding their biliary elimination. The proportion of administered ceftriaxone excreted by bile after each dose has been estimated at approximately 40% (21, 22), being 4 times higher than that of cefotaxime (23, 24). In their animal study, van Ogtrop and colleagues treated mice either with cefoperazone, ceftriaxone, cefepime or ceftazidime, whose intestinal elimination ranges from 0.2% to 37% of the administered dose (25). Although all 4 cephalosporins exhibited similar effects on Gram negative bacteria, some differences were observed: when higher doses were administered, the impact of antibiotics increased with the fractional intestinal elimination, in particular regarding colonization resistance. However, differences in antibacterial spectrum and dosing regimens of studied antibiotics in these animals might be part of these differences. In the CEREMI trial, we compared the impact of two 3rd generation cephalosporins at their clinical dose,

whose antibacterial spectrum has previously been reported to be similar, to analyse specifically the impact of pharmacokinetic differences (26).

Interestingly, fecal concentrations of both antibiotics were very low in included subjects. It had been reported previously that ceftriaxone excreted through the bile is microbiologically inactivated (19, 27). This might be due to the presence of endogenous beta-lactamases from microbial origin. Several authors reported that beta-lactam antibiotics can be inactivated by the enzymatic activity of the gut microbiota (28, 29). In particular, using a semiquantitative test for measuring the beta-lactamase activity in the fecal content, Leonard et al. observed that 2 of 6 volunteers treated by ceftriaxone exhibited undetectable levels of antibiotic when fecal beta-lactamase activity was detectable (30). This has been further illustrated by the administration of an oral beta-lactamase, which was shown to reduce fecal concentrations of ceftriaxone (31). This beta-lactamase activity of commensal bacteria might protect against selection of resistant Gram-negative enteric bacilli populations (32, 33).

Despite these low antibiotic concentrations, the intestinal microbiota was deeply affected by the 3-day course of both antibiotics, although no significant difference was found between groups. It is probable that antibiotics exert their effect on the upper part of the intestine, whereas we could only measure the concentrations of antibiotics in the fecal content. In addition, a majority of intestinal *Bacteroides* might not be affected by administered antibiotics, and replace suppressed Gram-negative enteric bacilli populations. This would thereby leave no room for selection of resistant Gram-negative enteric bacilli populations. To our knowledge, no data is however available for supporting this hypothesis.

The absence of difference regarding the selection of phenotypic resistance among Gram-negative enteric bacilli might be explained by the design of the trial, which was performed in healthy volunteers. This community setting probably results in a low selective pressure, thereby limiting the risk of colonisation with resistant bacterial strains. The exposure to the healthcare system of patients requiring intravenous antibiotic therapy might be an additional factor contributing to the differences observed in existing ecological studies (11, 12).

More startling is the similarity of both antibiotics' impact on the microbiome. This observation might be further explained by considering the dose administered of each antibiotic. Total daily dose of cefotaxime is indeed 3 times higher than that of ceftriaxone. Using known data on biliary elimination of the 2 antibiotics, we can infer that the daily amount of antibiotic reaching the gut is roughly similar for both antibiotics (400 mg for ceftriaxone 1g/day, and 300 mg for cefotaxime 1g/8h). It is noteworthy that despite being administered with lower daily dose, the pharmacodynamics profile of ceftriaxone, as measured by the fractional time during which the free plasma concentration of cefotaxime is above the susceptibility breakpoint of *Enterobacteriaceae*, is more favorable than that of cefotaxime, with a

similar impact on the intestinal microbiota. This suggests that ceftriaxone might be better suited to reduce the overall consumption of antibiotics, and thereby the environmental spread of antibiotic residues.

This work has some limitations. First, we included only healthy volunteers. Our results might thus not reflect the global effect of both antibiotics on the intestinal microbiota when treating infected patients. Here, we aimed at analysing the intrinsic impact of ceftriaxone and cefotaxime, avoiding confounding factors such as repeated antibiotic treatments or frequent contact with the healthcare system. Another limitation is the absence of data regarding the beta-lactamase activity in feces. This would have provided further details on the results regarding fecal concentrations of antibiotics. This activity might also be used as a proxy of the exposure of the intestinal microbiota to beta-lactam antibiotics. Of note, the prevalence of Gram-negative bacilli is somewhat lower than expected (34). It might have been underestimated as we chose to use a selective agar (Drigalski) in order to quantify total enterobacteria from fecal microbiota in a reproducible way, which is difficult without using selective media. Furthermore, analyses were performed on the same medium for all patients which allowed to avoid any differential bias.

In spite of these limitations, this trial provides further insights into the comparison of ceftriaxone and cefotaxime impact on the intestinal microbiota. Under our experimental conditions, our results suggest that both antibiotics exert a pronounced impact on the intestinal microbiota, extending what is already known on their antibacterial spectrum for cultivable bacteria. This was observed while total daily dose of cefotaxime administered was 3 times higher than that of ceftriaxone, as requested in clinical practice. Deeper analysis of the resistance within the microbiota, such as the use of shotgun metagenomics for studying the fecal content in resistance-conferring genes or the use of the recently released targeted sequence capture ResCap (35), would be of great value in order to draw a complete picture of the possible difference of selective pressure between these 2 antibiotics.

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Conflict of interest

All authors reported no conflict of interest.

Author Participation

CB, NG, LM, ST, AA, FM, XD conceived the study. CB, ED, AA, FM, XD obtained funding. CB, MB, LA, XD included the subjects. NG, CDH, AA performed the phenotypic analyses. ABN, KB, MM, OT, ED performed the 16S rRNA gene profiling. KB, OC, ED performed the molecular analysis of the resistant strains. LM performed the pharmacologic analyses. CB, ML, FM performed the statistical analyses. CB, NG, FM, XD wrote the first draft of the manuscript. All author approved the final version of the manuscript.

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Figures

Figure 1. Evolution of the counts of the studied microorganisms in the fecal samples of the 22 included subjects treated by ceftriaxone (n=11, blue) or cefotaxime (n=11, green). Studied microorganisms included 3rd generation cephalosporin Gram-negative enteric bacilli (measured on AES plates, panel A or ChromID ESBL plates, panel B), total Gram-negative enteric bacilli (panel C), *Enterococcus* spp. (panel D), *S. aureus* (panel E), *P. aeruginosa* (panel F), *C. difficile* (panel G) and Yeasts (panel H). The light grey zone represents the treatment period. Thin lines represent individual values, and thick lines represent the median change from baseline of the log-counts at each time.

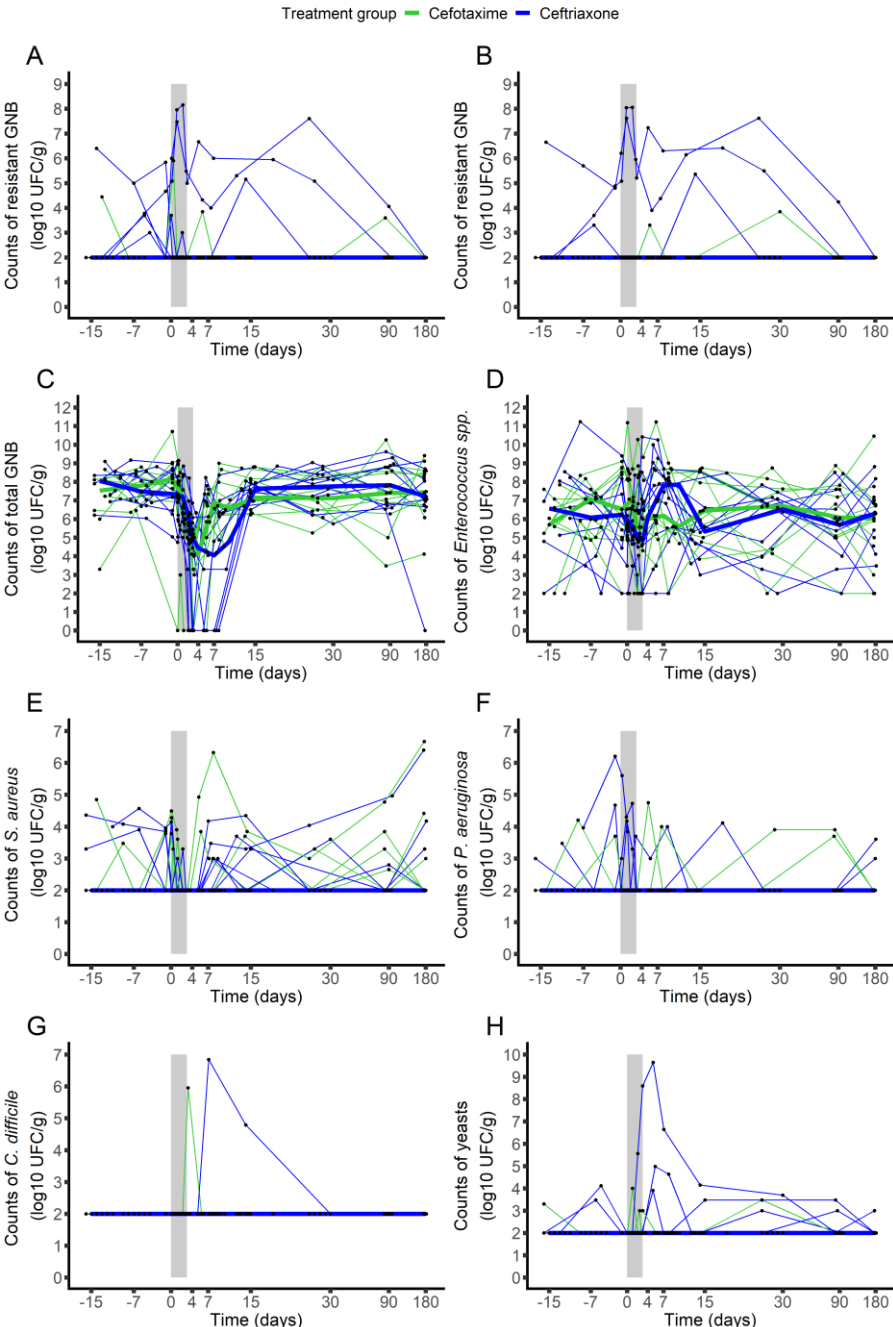


Figure 2. Boxplots of the change from baseline of relative abundances (in %) of the main bacterial phyla at day 4, day 7, day 10, day 30 and day 180 in the 22 included subjects treated by ceftriaxone (n=11, panel A) or cefotaxime (n=11, panel B). The boxes present the 25th and 75th percentiles and the horizontal black bar report the median value, while whiskers report 10th and 90th percentiles.

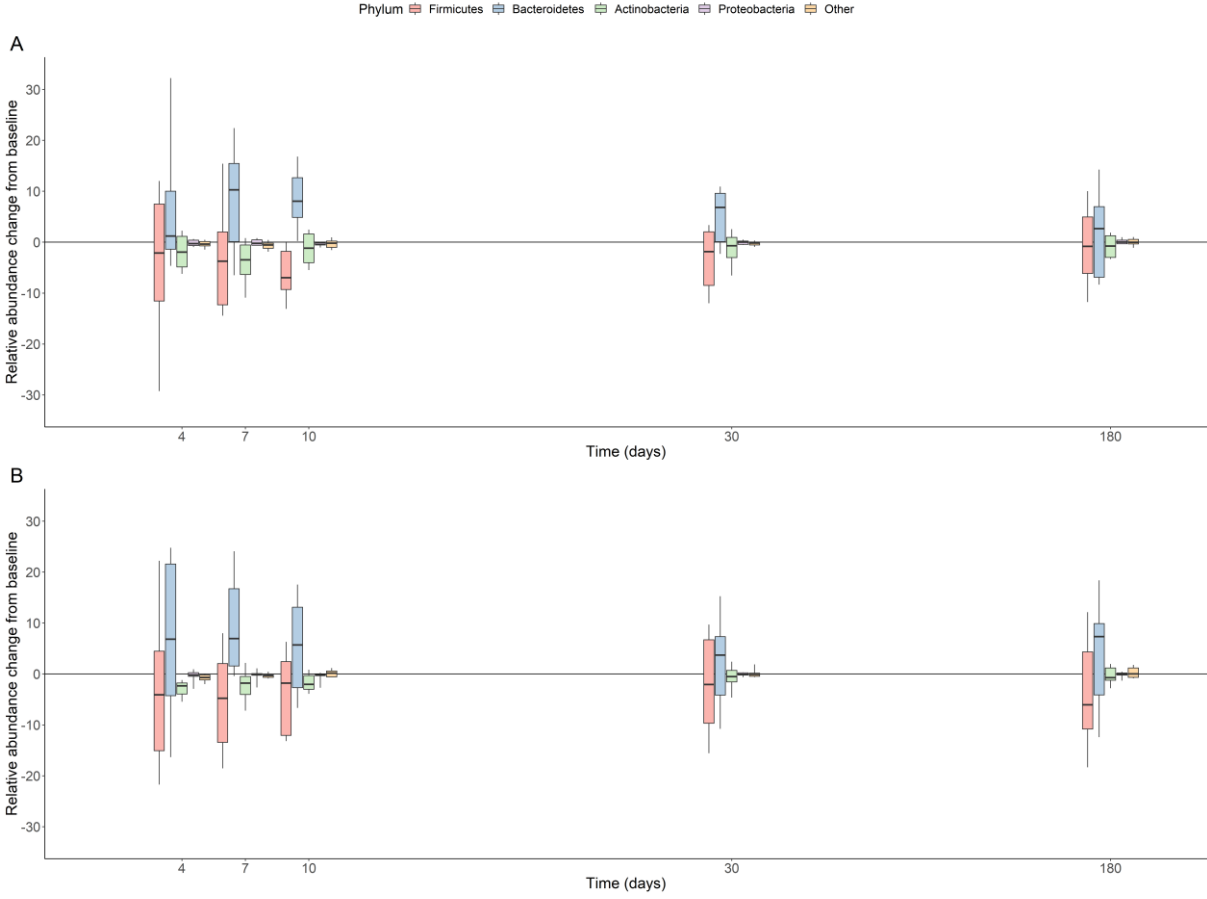


Figure 3. Evolution of the change from baseline of the Shannon index (panel A), change from baseline of the number of OTUs (panel B), Bray-Curtis dissimilarity from baseline (panel C) and unweighted UniFrac distance from baseline (panel D) in the fecal samples of the 22 included subjects treated by ceftriaxone (n=11, blue) or cefotaxime (n=11, green). The light grey zone represents the treatment period. Thin lines represent individual values, and thick lines represent the mean of the index values at each time. In panels C and D, horizontal black lines represent the median value of the β -diversity index for each subject between the samples collected at day -15 and the sample collected at day -1.

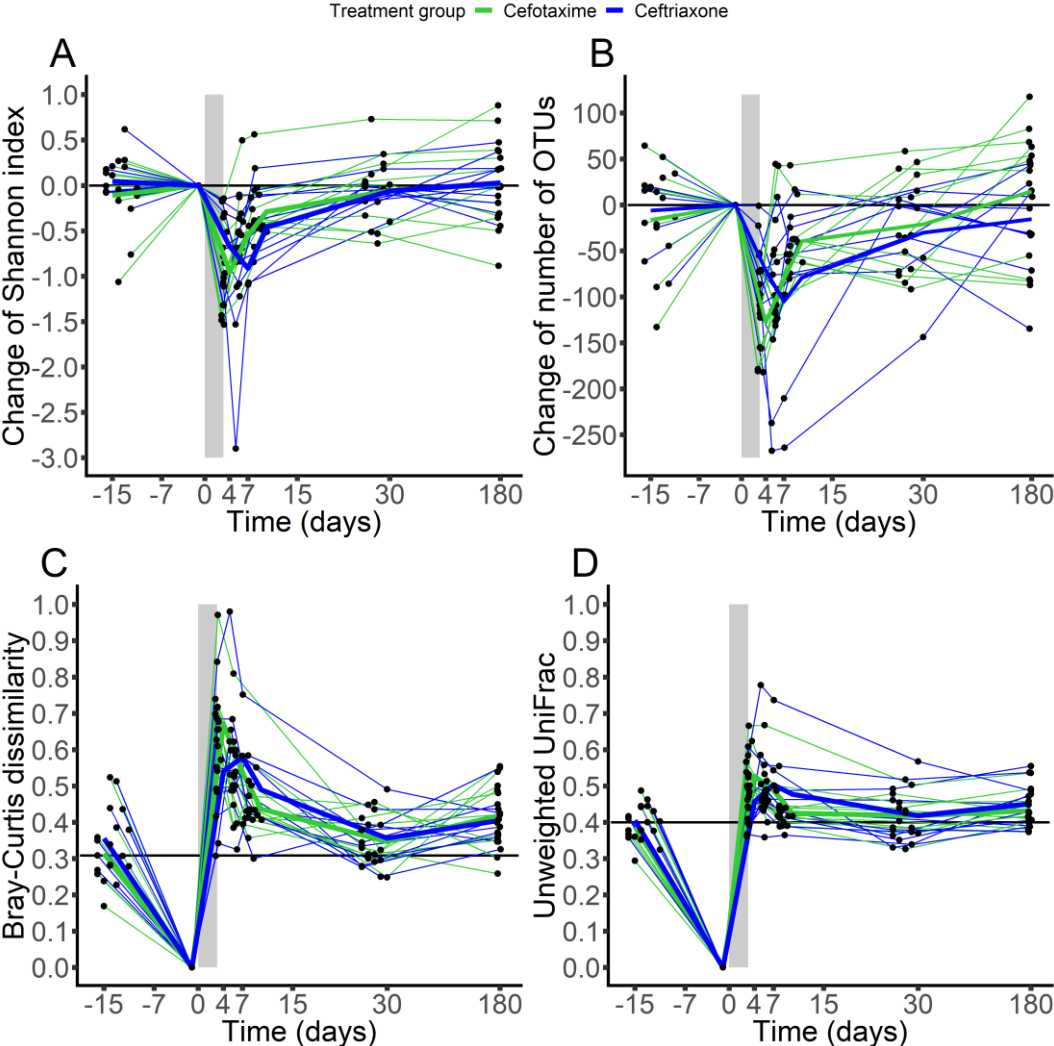


Table 1. Baseline characteristics of the 22 included subjects. Baseline counts of microorganisms were computed as the arithmetic mean of the log₁₀ counts observed on available pre-treatment samples at days -15, -7 and -1. Data are presented as median (min; max).

	Ceftriaxone (n=11)	Cefotaxime (n=11)
Clinical characteristics		
Gender : men (%)	2 (18)	4 (36)
Age (years)	30 (24; 55)	24 (18; 61)
Total body weight (kg)	72.0 (57.9; 78.4)	63.4 (49.9; 87.0)
Body mass index (kg/m ²)	24.6 (20.0; 28.7)	22.0 (18.6; 28.9)
Counts of microorganisms in feces at baseline (log₁₀ CFU/g)		
Resistant enterobacteria (AES agar)	<2.0 (<2.0; 5.4)	<2.0 (<2.0; 2.8)
Resistant enterobacteria (ChromID ESBL agar)	<2.0 (<2.0; 5.7)	<2.0 (<2.0; <2.0)
Total enterobacteria	7.7 (5.8; 8.7)	7.8 (3.3; 9.1)
<i>Enterococcus</i> spp.	6.2 (3.4; 9.2)	6.7 (3.3; 7.8)
<i>S. aureus</i>	<2.0 (<2.0; 4.2)	<2.0 (<2.0; 3.0)
<i>P. aeruginosa</i>	<2.0 (<2.0; 5.1)	<2.0 (<2.0; 2.7)
<i>C. difficile</i>	<2.0 (<2.0; <2.0)	<2.0 (<2.0; <2.0)
Yeasts	<2.0 (<2.0; 2.7)	<2.0 (<2.0; 2.4)
Indices of α-diversity at day -1		
Shannon index	3.9 (3.2; 4.3)	4.0 (2.8; 4.3)
Number of OTUs	451 (308; 591)	451 (267; 521)
Indices of β-diversity between day -1 and day -15		
Bray-Curtis dissimilarity	0.35 (0.23; 0.51)	0.29 (0.17; 0.52)
unweighted UniFrac distance	0.41 (0.32; 0.46)	0.38 (0.29; 0.49)
Relative abundance (in %) of the main bacterial phyla at day -1		
<i>Actinobacteria</i>	4.7 (1.3; 13.5)	4.3 (2.1; 10.0)
<i>Bacteroidetes</i>	34.1 (23.2; 52.2)	40.2 (24.2; 60.4)
<i>Firmicutes</i>	55.7 (43.9; 69.1)	54.1 (32.7; 68.2)
<i>Proteobacteria</i>	0.9 (0.3; 1.6)	0.6 (0.2; 7.4)
Other phyla	1.2 (0.3; 2.6)	1.4 (0.7; 3.2)

Table 2. Derived pharmacokinetic endpoints of ceftriaxone and cefotaxime for the 11 included subjects of each treatment group, derived from the estimated individual pharmacokinetic parameters.

Parameter	Ceftriaxone (n=11)	Cefotaxime (n=11)
$fAUC_{0-24,ss}$ (mg.h/L)	83.8 (59.0; 105.3)	121.5 (83.6; 187.0)
fC_{max} (mg/L)	13.5 (10.8; 14.9)	40.6 (29.4; 55.9)
fC_{min} (mg/L)	0.9 (0.4; 1.5)	0.1 (0.1; 0.2)
$fT>1$ (%)	94.2 (68.6; 100)	53.0 (44.5; 66.7)

Table 3. Area under the curve (AUC) of the variation from baseline of the counts of studied microorganisms between baseline and day 7 or day 15 and of bacterial diversity indices between day -1 and day 30 in the fecal samples of the 22 included subjects. Baseline counts were computed as the arithmetic mean of the log₁₀ counts observed on pre-treatment samples. AUC_{D0D15} (respectively AUC_{D-1D30}) were normalized by the actual time observed between baseline and day 15 (respectively day 30). Data are presented as median (min; max). P-values refer to the result of non-parametric Wilcoxon test.

	Ceftriaxone (n=11)	Cefotaxime (n=11)	p-value
AUC_{D0D7} of raw bacterial counts (log₁₀ CFU.day/g)			
Resistant enterobacteria (AES agar)	0.0 (0.0; 23.4)	0.0 (0.0; 2.7)	0.40
Resistant enterobacteria (ChromID ESBL agar)	0.0 (0.0; 24.3)	0.0 (0.0; 1.5)	0.28
AUC_{D0D7} of counts change from baseline (log₁₀ CFU.day/g)			
Total enterobacteria	-14.8 (-30.0; -7.0)	-11.2 (-26.0; -6.2)	0.26
<i>Enterococcus</i> spp.	-0.3 (-8.6; 2.6)	-0.9 (-3.5; 4.1)	0.56
<i>S. aureus</i>	0.0 (-9.7; 1.3)	0.0 (-2.7; 1.7)	0.23
<i>P. aeruginosa</i>	0.0 (-8.4; 5.5)	0.0 (-4.0; 2.9)	0.87
<i>C. difficile</i>	0.0 (0.0; 0.0)	0.0 (0.0; 7.0)	0.36
Yeasts	0.0 (-3.9; 18.4)	0.0 (-2.5; 2.4)	0.85
AUC_{D0D15} of counts change from baseline (log₁₀ CFU.day/g)			
Resistant enterobacteria (AES agar)	0.0 (-50.7; 40.3)	0.0 (-12.2; 4.9)	>0.99
Resistant enterobacteria (ChromID ESBL agar)	0.0 (-6.5; 44.6)	0.0 (0.0; 6.0)	0.79
Total enterobacteria	-30.7 (-79.2; -12.4)	-21.8 (-37.5; 16.4)	0.088
<i>Enterococcus</i> spp.	7.6 (-38.1; 39.5)	6.2 (-31.5; 19.3)	0.75
<i>S. aureus</i>	1.2 (-18.5; 6.6)	0.0 (-7.3; 21.2)	0.82
<i>P. aeruginosa</i>	0.0 (-25.5; 13.6)	0.0 (-8.4; 7.0)	0.69
<i>C. difficile</i>	0.0 (0.0; 33.7)	0.0 (0.0; 7.5)	>0.99
Yeasts	0.0 (-10.5; 53.7)	0.0 (-6.5; 2.5)	0.46
AUC_{D-1D30} of change from baseline of diversity indices (unit.day)			
Shannon diversity index	-12.0 (-26.6; 2.4)	-10.5 (-21.6; 15.1)	0.85
Number of OTUs	-1391 (-5708; 114)	-1816 (-2677; 907)	>0.99
Bray-Curtis dissimilarity	12.2 (10.3; 19.1)	12.2 (10.7; 17.6)	0.95
Unweighted UniFrac distance	12.1 (10.0; 18.7)	12.5 (10.9; 16.9)	0.75

Table 4. Emergence of 3rd generation cephalosporins resistant Gram-negative enteric bacilli or noncommensal microorganisms of the intestinal tract between day 1 and day 15 in the 22 included subjects. Only subjects who were not colonized before the beginning of treatment were included in the analysis. For resistant Gram-negative enteric bacilli, results from either AES or ChromID ESBL agars plates were considered for definition of the emergence of resistance. Data are presented as n (%). P-values refer to the result of non-parametric Fisher exact test.

	Ceftriaxone		Cefotaxime		p-value
	Subjects at risk	Subjects colonized	Subjects at risk	Subjects colonized	
Resistant Gram-negative enteric bacilli	6	1 (16.7%)	10	2 (20%)	>0.99
<i>S. aureus</i>	7	6 (85.7%)	8	3 (37.5%)	0.12
<i>P. aeruginosa</i>	8	1 (12.5%)	9	1 (11.1%)	>0.99
<i>C. difficile</i>	11	1 (9.1%)	11	1 (9.1%)	>0.99
Yeasts	9	3 (33.3%)	10	3 (30%)	>0.99

Supplementary Material

Supplementary text S1. Supplementary methods

Analysis of 3rd generation cephalosporins resistant Gram-negative enteric bacilli

In a first step, all *E. coli* strains resistant to 3rd generation cephalosporins were phylogrouped and O-typed by PCR-based approaches as in (1, 2). According to the result, the belonging to the ST131/506 was also performed as in (3, 4). In a second step, one to four strains per subject, as well as an *Enterobacter cloacae* strain, were sequenced on an Illumina MiSeq® (2 X 300 bp). Assembly was performed with SPAdes, version 3.10.1 using standard parameters (5). Multilocus sequence type and O-and H-type determination and resistome were determined on the Center for Genomic Epidemiology platform (www.genomicepidemiology.org).

16S rRNA gene profiling of the intestinal microbiota

Microbial DNA was extracted using QIAamp DNA stool Kit (Qiagen, Germany). The V4 region of the 16S rRNA gene was then amplified using primers selected from (6). Their sequences were TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCA GCM GCC GCG GTA A for primer 515f and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACN VGG GTW TCT for primer 806r. The PCR amplicons (292 bp) were sequenced on an Illumina MiSeq® platform according to the manufacturer's specifications (Illumina, USA). Demultiplexing and quality filtering were performed using mothur (7). No mismatch was allowed in the primers sequences in both forward and reverse primers, and quality-filtering was performed by truncating bases at the 3' end with Phred quality score <25. Paired-end read assembly was then performed with 100% overlap identity.

Following these pre-processing steps, chimera sequences were detected and eliminated using Vsearch (8). Then, clustering of similar sequences (97% identity threshold for an affiliation at the genus level) was performed through an open-reference OTU picking process and complete-linkage method, finally creating groups of sequences or "Operational Taxonomic Units" (OTUs). A final OTU cleaning step corresponding to the elimination of singletons and doubletons was performed. Diversity indices were computed for each sample after rarefaction of the data (59,742 sequences allowed an exhaustive description of the bacterial diversity).

Pharmacokinetic analysis of plasma concentrations of antibiotics

For the analysis of total plasma concentration of ceftriaxone and cefotaxime, 2 separate population pharmacokinetic analyses were performed using the Stochastic Approximation Expectation Minimization (SAEM) algorithm in Monolix v4.2 (Lixoft, Orsay, France, available at <http://www.lixoft.com>), which handles concentrations below the limit of quantification to improve parameters estimation (9). Both one- and two-compartment(s) models with first order elimination were tested, and parameters to be estimated were V, the central volume of distribution and k, the

first-order elimination rate. For two-compartment models, we also estimated k_{12} and k_{21} , the transfer rates between central and peripheral compartments.

We used an exponential random effects model for each pharmacokinetic parameter. We assumed the random effects to have a normal distribution with a mean of 0 and a variance of ω^2 . The residual error model was supposed to be additive, proportional or combined, with σ_{inter} being the standard deviation of the additive component and σ_{slope} the standard deviation of the proportional component.

Evaluation of the final model was conducted using graphical methods. Basic goodness-of-fit plots were used, as well as the individual weighted residuals (IWRES), the normalized prediction distribution errors (NPDE) over time and the visual predictive check (VPC). NPDE and VPC were generated using 500 Monte Carlo simulations. The best model was chosen using the Bayesian information criteria (BIC), derived for each model from the computation of likelihood by importance sampling (10).

Individual pharmacokinetic parameters were estimated as the maximum of the *a posteriori* distribution, also called empirical Bayes estimates, and used to predict individual pharmacokinetic profiles of included subjects.

Supplementary Table S1. Estimated population parameters and their relative standard errors (r.s.e) for the pharmacokinetic models of ceftriaxone and cefotaxime in the plasma. The models were separately fitted on the data from the 11 subjects treated by ceftriaxone and on the data from the 11 subjects treated by cefotaxime. V is the volume of distribution; k is the extraintestinal elimination rate from the central compartment; k_{12} and k_{21} are the transfer rates between the central compartment and the peripheral compartment; σ_{slope} is the proportional component of the residual error.

Parameter	Ceftriaxone		Cefotaxime	
	Fixed effects (r.s.e. %)	Standard deviation of the exponential random effects (r.s.e. %)	Fixed effects (r.s.e. %)	Standard deviation of the exponential random effects (r.s.e. %)
V (L)	7.4 (4)	0.11 (24)	8.7 (13)	0.19 (32)
k (/h)	0.16 (6)	0.18 (22)	1.69 (10)	0.07 (38)
k_{12} (/h)	0.35 (13)	- (fixed)	0.87 (30)	- (fixed)
k_{21} (/h)	0.54 (9)	- (fixed)	1.35 (13)	- (fixed)
σ_{slope}		0.05 (11)		0.19 (12)

Supplementary Table S2. Main phenotypic and genotypic characteristics of the 3rd-generation cephalosporin-resistant Gram-negative enteric bacilli in the 22 included subjects. Phylogroup, sequence type 131/506 or 10 and O-type were determined in all *E. coli* strains by PCR-based assays as in (1-4). Sequenced strains are presented in bold fonts. ND, not determined.

Strain ID	Treatment group	Sampling		Species	Antibioresistance phenotype*	Phylogroup	ST		Serotype	Molecular support of resistance
		Subject	time (day)				Warwick**	Pasteur Institute***		
E1	ceftriaxone	1	-15	<i>E. coli</i>	ESBL	B2	131	506	O16:H5	CTX-M-55
E3	ceftriaxone	1	-7	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E5	ceftriaxone	1	-1	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E7	ceftriaxone	1	1	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E9	ceftriaxone	1	2	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E8	ceftriaxone	1	4	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E10	ceftriaxone	1	7	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E12	ceftriaxone	1	10	<i>E. coli</i>	ESBL	B2	131	506	O16:H5	CTX-M-55
E13	ceftriaxone	1	15	<i>E. coli</i>	ESBL	B2	131	506	O16	ND
E14	ceftriaxone	1	30	<i>E. coli</i>	ESBL	B2	131	506	O16:H5	CTX-M-55
E15	ceftriaxone	1	90	<i>E. coli</i>	ESBL	B2	131	567	O16:H5	CTX-M-55
E11	cefotaxime	2	7	<i>E. coli</i>	pCASE	F	117	539	O8:H4	CMY-2
E2	cefotaxime	3	-15	<i>E. coli</i>	ESBL	B2	131	506	O16:H5	CTX-M-55
E4	ceftriaxone	6	-7	<i>Hafnia alvei</i>	dCASE	ND	ND	ND	ND	ND
E17	ceftriaxone	6	-1	<i>H. alvei</i>	dCASE	ND	ND	ND	ND	ND
E16	ceftriaxone	9	15	<i>E. coli</i>	ESBL	A	46	398	O9:H4	CTX-M-15
E19	cefotaxime	17	7	<i>Enterobacter cloacae</i>	ESBL	ND	ND	ND	ND	CTX-M-15
E18	cefotaxime	17	30	<i>E. coli</i>	ESBL	B1	58	24	O9:H25	CTX-M-1
E22	ceftriaxone	18	-7	<i>E. coli</i>	pCASE	A	10	913	O8:H17	CMY-4
E23	ceftriaxone	18	1	<i>E. coli</i>	pCASE	A	10	ND	O8	ND
E21	ceftriaxone	18	3	<i>E. coli</i>	pCASE	A	10	913	O8:H17	CMY-4
E20	ceftriaxone	22	-7	<i>Citrobacter freundii</i>	dCASE	ND	ND	ND	ND	ND
E25	ceftriaxone	28	-7	<i>E. coli</i>	ESBL	A	10	2	O71:H48	CTX-M-1
E26	ceftriaxone	28	-1	<i>E. coli</i>	ESBL	A	10	ND	O73	ND
E27	ceftriaxone	28	1	<i>E. coli</i>	ESBL	A	10	ND	O73	ND
E33	ceftriaxone	28	3	<i>E. coli</i>	ESBL	A	10	ND	O73	ND
E34	ceftriaxone	28	4	<i>E. coli</i>	ESBL	A	10	ND	O73	ND

E29	ceftriaxone	28	7	<i>E. coli</i>	ESBL	A	10	ND	O73	ND
E31	ceftriaxone	28	10	<i>E. coli</i>	ESBL	A	10	ND	O73	ND
E32	ceftriaxone	28	15	<i>E. coli</i>	ESBL	A	10	914	O73:H31	CTX-M-1
E30	ceftriaxone	28	30	<i>E. coli</i>	ESBL	A	10	914	O73:H31	CTX-M-1

* ESBL, extended spectrum beta-lactamase; pCASE, plasmid-encoded cephalosporinase; dCASE, derepressed cephalosporinase

** <https://enterobase.warwick.ac.uk/species/index/ecoli>

*** <https://www.pasteur.fr/fr/sante-publique/CNR/les-cnr/escherichia-coli-shigella-salmonella>

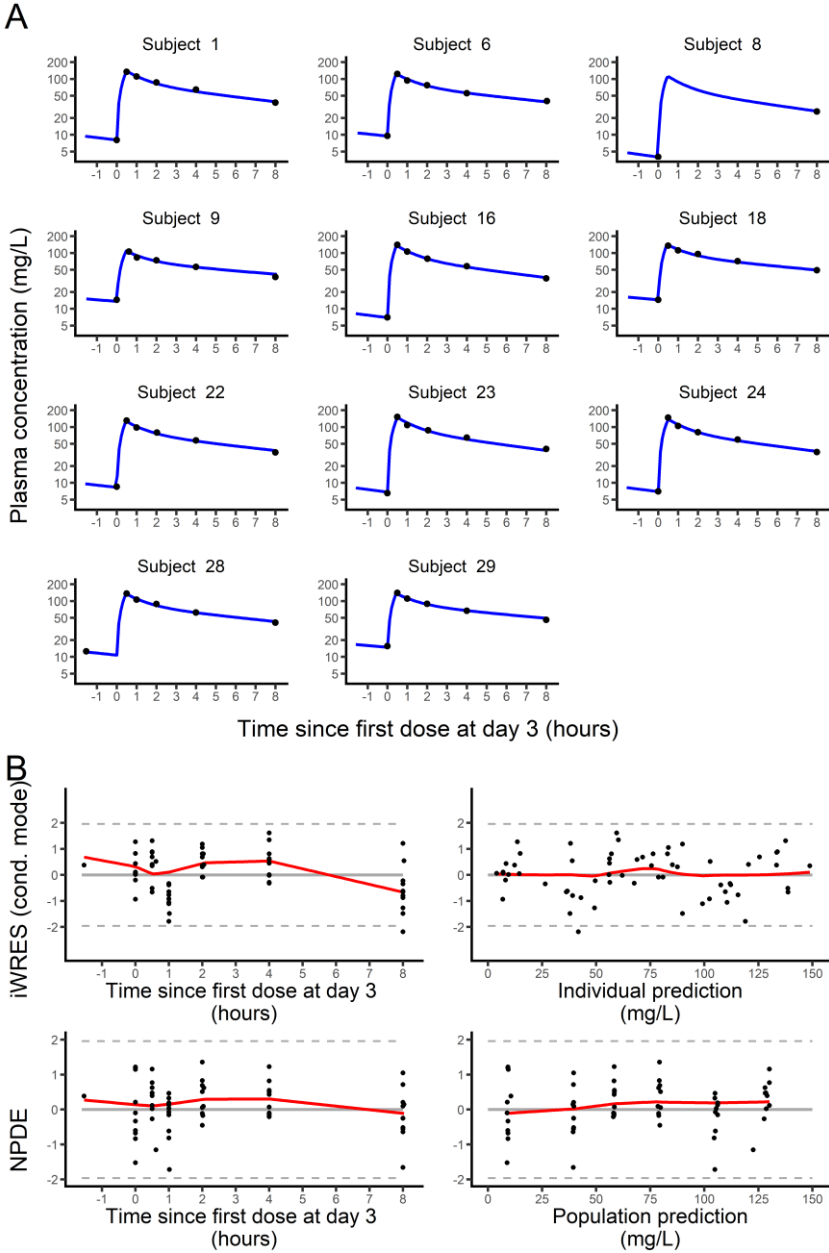
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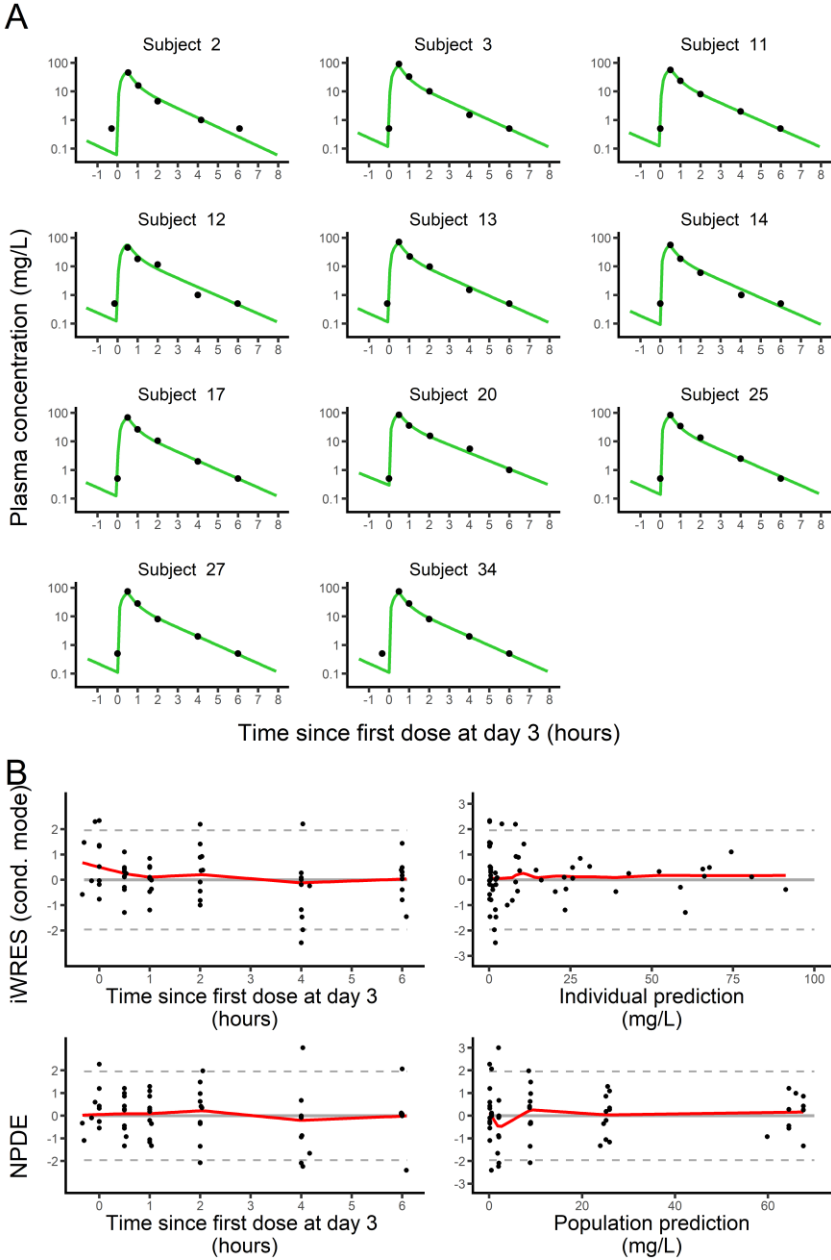
Supplementary Table S3. Spearman rank correlation coefficients of the pharmacokinetic endpoints of ceftriaxone and cefotaxime and of the AUC_{D-1D30} of change from baseline of studied indices of bacterial diversity for the 11 included subjects of each treatment group. P-values refer to the comparison to 0 of the correlation coefficients and are presented in brackets.

	Pharmacokinetic endpoints			
	$fAUC_{0-24,ss}$	fC_{max}	fC_{min}	$fT>1$
Ceftriaxone (n=11)				
Shannon diversity index	0.0 (0.98)	-0.2 (0.58)	-0.1 (0.86)	0.0 (0.91)
Number of OTUs	0.1 (0.80)	-0.1 (0.86)	0.1 (0.71)	0.1 (0.69)
Bray-Curtis dissimilarity	-0.2 (0.56)	0.0 (0.99)	0.0 (0.90)	-0.1 (0.86)
Unweighted UniFrac distance	-0.3 (0.37)	0.0 (0.95)	-0.2 (0.54)	-0.3 (0.36)
Cefotaxime (n=11)				
Shannon diversity index	-0.2 (0.63)	-0.2 (0.63)	-0.2 (0.58)	-0.1 (0.71)
Number of OTUs	-0.3 (0.36)	-0.3 (0.36)	-0.1 (0.78)	-0.3 (0.41)
Bray-Curtis dissimilarity	-0.1 (0.88)	-0.1 (0.88)	0.0 (0.95)	-0.1 (0.80)
Unweighted UniFrac distance	0.5 (0.12)	0.5 (0.12)	0.2 (0.52)	0.5 (0.12)

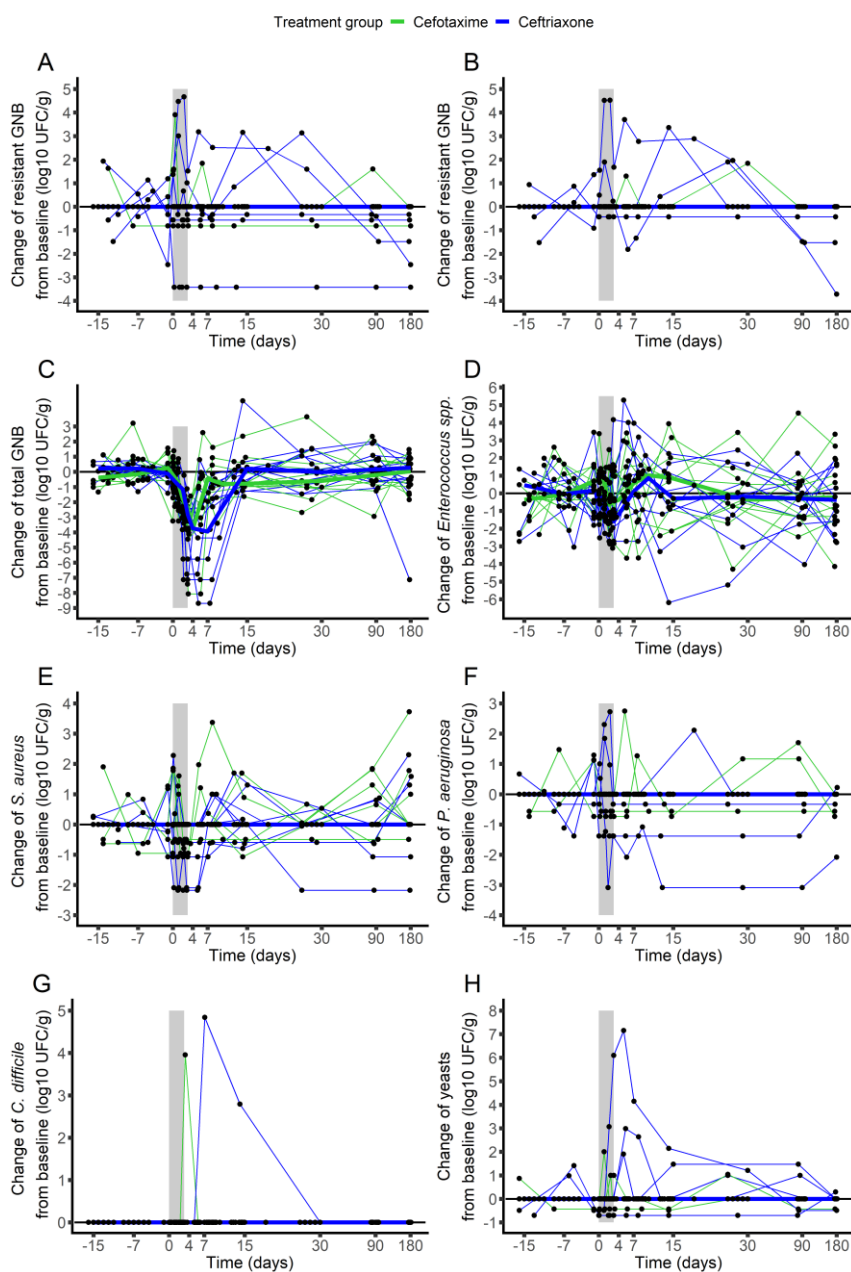
Supplementary Figure S1. Goodness of fit plots for the pharmacokinetic model of ceftriaxone in plasma. Panel A presents the individual fits of the model for the plasma total concentration of ceftriaxone. Black dots represent observed moxifloxacin concentrations. Orange curves represent the individual pharmacokinetic profiles predicted by the model using estimated individual parameters. Panel B presents the plots of the individual weighted residuals (iWRES) and normalized prediction distribution errors (NPDE) over time (left) or model predictions (right) for the total plasma concentrations. The iWRES and NPDE are shown as black points, and spline lines are also added as red curves.



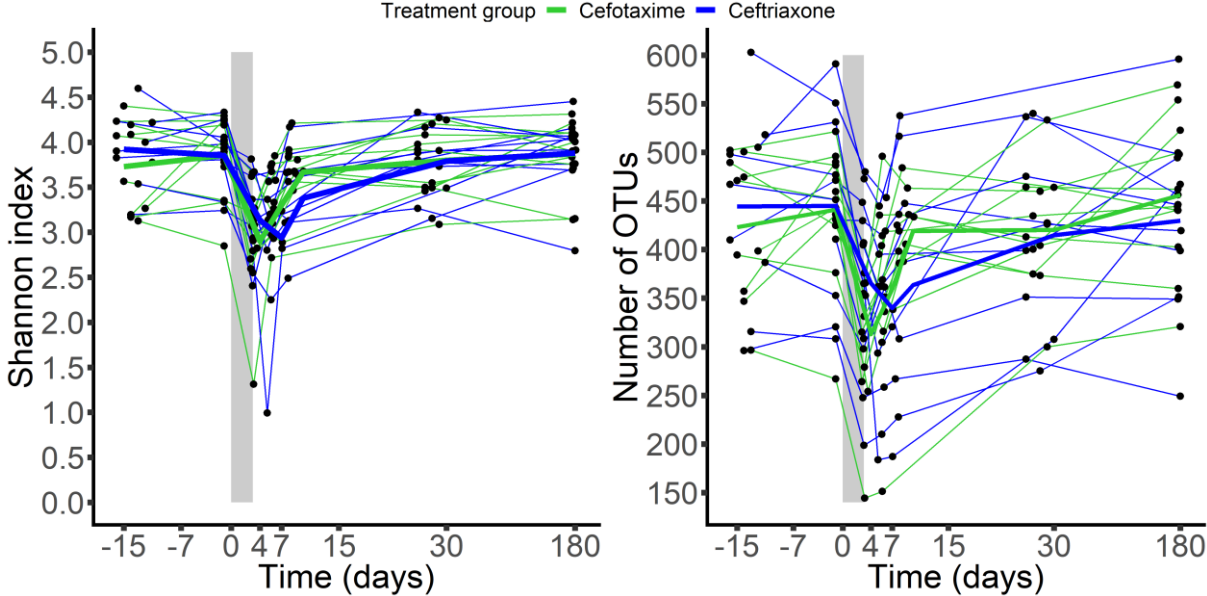
Supplementary Figure S2. Goodness of fit plots for the pharmacokinetic model of cefotaxime in plasma. Panel A presents the individual fits of the model for the plasma total concentration of cefotaxime. Black dots represent observed moxifloxacin concentrations. Green curves represent the individual pharmacokinetic profiles predicted by the model using estimated individual parameters. Panel B presents the plots of the individual weighted residuals (iWRES) and normalized prediction distribution errors (NPDE) over time (left) or model predictions (right) for the total plasma concentrations. The iWRES and NPDE are shown as black points, and spline lines are also added as red curves.



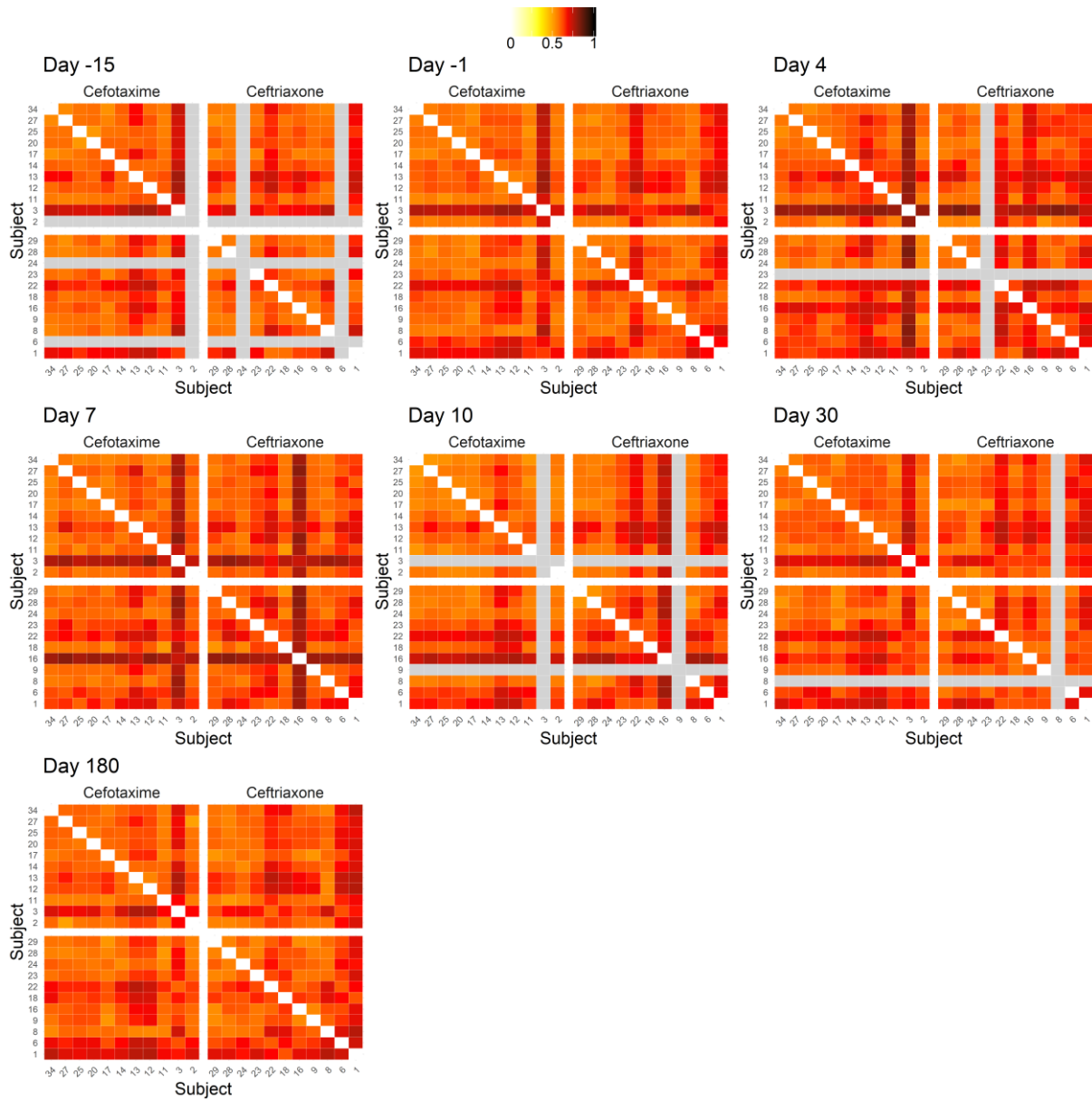
Supplementary Figure S3. Evolution of the change from baseline of the counts of the studied microorganisms in the fecal samples of the 22 included subjects treated by ceftriaxone (n=11, blue) or cefotaxime (n=11, green). Studied microorganisms included 3rd generation cephalosporin Gram-negative enteric bacilli (measured on AES plates, panel A or ChromID ESBL plates, panel B), total Gram-negative enteric bacilli (panel C), *Enterococcus* spp. (panel D), *S. aureus* (panel E), *P. aeruginosa* (panel F), *C. difficile* (panel G) and Yeasts (panel H). The light grey zone represents the treatment period. Thin lines represent individual values, and thick lines represent the median change from baseline of the log-counts at each time. For graphical reasons, baseline counts are represented at day 0. GNB, Gram-negative enteric bacilli.



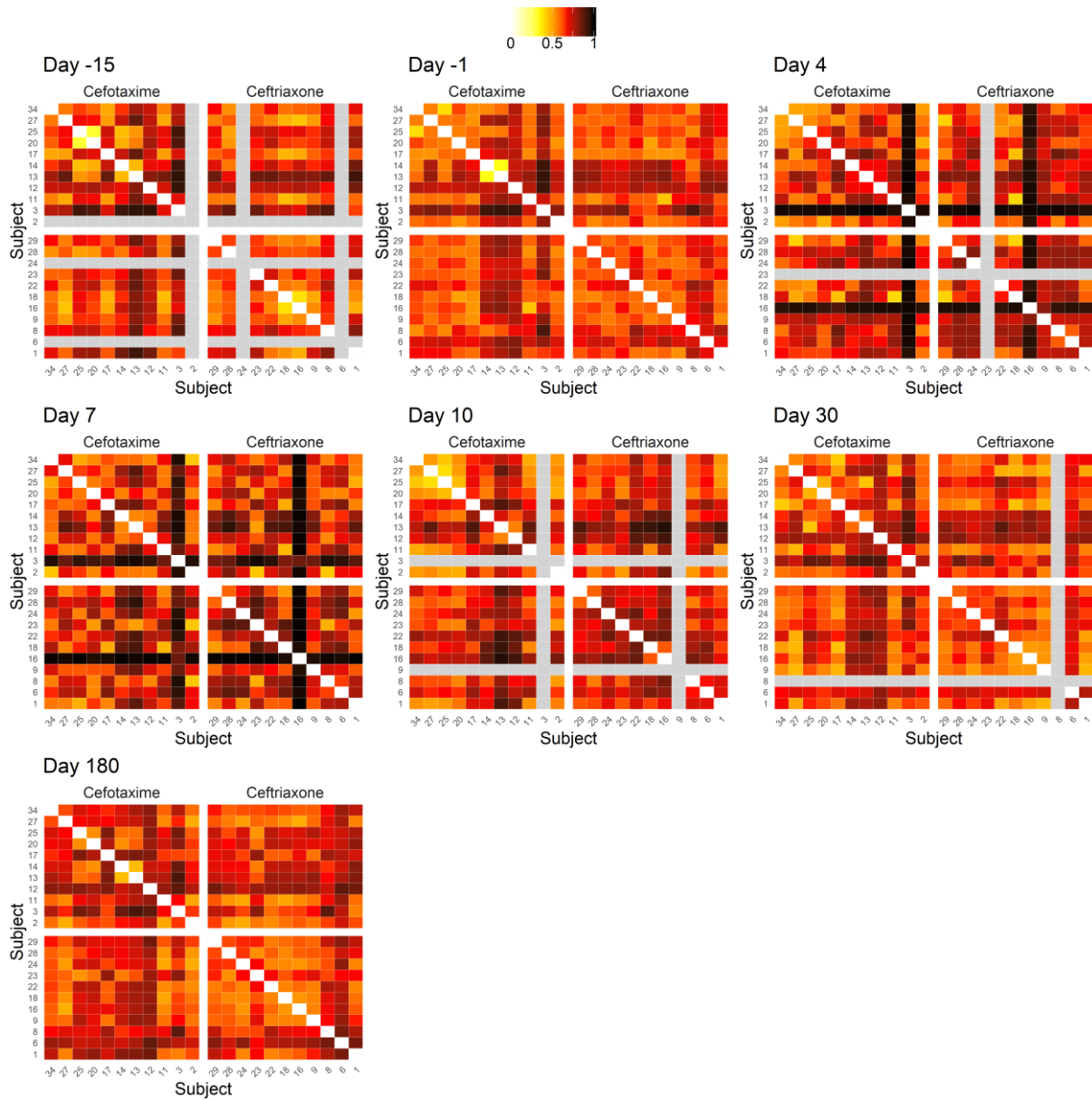
Supplementary Figure S4. Evolution of the Shannon index (panel A) and of the number of OTUs (panel B) in the fecal samples of the 22 included subjects treated by ceftriaxone (n=11, blue) or cefotaxime (n=11, green). The light grey zone represents the treatment period. Thin lines represent individual values, and thick lines represent the mean of the index values at each time.



Supplementary Figure S5. Heatmap of the unweighted UniFrac distances matrix at each sampling time for the 22 included subjects. Missing samples are presented in lightgrey.



Supplementary Figure S6. Heatmap of the Bray-Curtis dissimilarity matrix at each sampling time for the 22 included subjects. Missing samples are presented as lightgrey.



Supplementary Figure S7. Heatmap of the intraindividual matrices of β -diversity indices at the various sampling times: unweighted UniFrac distance (panels A & B) and Bray-Curtis dissimilarity (panels C & D) between day -15 and day 180 for the subjects treated with ceftriaxone (n=11, panel A & C) or cefotaxime (n=11, panel B & D). Missing samples are presented in lightgrey.

