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Impact of Ceftiofur Injection on Gut Microbiota and *Escherichia coli* Resistance in Pigs

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Resistance to extended-spectrum cephalosporins (ESCs) is an important health concern. Here, we studied the impact of the administration of a long-acting form of ceftiofur on the pig gut microbiota and ESC resistance in *Escherichia coli*. Pigs were orally inoculated with an ESC-resistant *E. coli* M63 strain harboring a conjugative plasmid carrying a gene conferring resistance, $bla_{CTX\text{-}M\text{-}1}$. On the same day, they were given or not a unique injection of ceftiofur. Fecal microbiota were studied using quantitative PCR analysis of the main bacterial groups and quantification of short-chain fatty acids. *E. coli* and ESC-resistant *E. coli* were determined by culture methods, and the ESC-resistant *E. coli* isolates were characterized. The copies of the $bla_{CTX\text{-}M\text{-}1}$ gene were quantified. After ceftiofur injection, the main change in gut microbiota was the significant but transitory decrease in the *E. coli* population. Acetate and butyrate levels were significantly lower in the treated group. In all inoculated groups, *E. coli* M63 persisted in most pigs, and the $bla_{CTX\text{-}M\text{-}1}$ gene was transferred to other *E. coli*. Culture and PCR results showed that the ceftiofur-treated group shed significantly more resistant strains 1 and 3 days after ESC injection. Thereafter, on most dates, there were no differences between the groups, but notably, one pig in the nontreated group regularly excreted very high numbers of ESC-resistant *E. coli*, probably leading to a higher contamination level in its pen. In conclusion, the use of ESCs, and also the presence of high-shedding animals, are important features in the spread of ESC resistance.

Resistance to extended-spectrum cephalosporins (ESCs) is a major public health concern. ESC-resistant *Enterobacteriaceae* are often resistant to other families of antimicrobials, leading to therapeutic problems, and ESCs are classified as "critically important antimicrobials" in human medicine by the World Health Organization (1). ESC-resistant *Enterobacteriaceae* are prevalent in food-producing animals in various countries (2), and these resistant strains are sometimes present at alarming rates in the gut microbiota of animals (3, 4). In pigs, as in humans, the most frequently reported extended-spectrum β-lactamases that confer resistance are the CTX-M group (5), which are encoded by genes present on conjugative plasmids. In some countries, third- and fourth-generation cephalosporins are authorized for use in pigs to fight respiratory diseases associated with *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis*, and *Streptococcus suis*, mastitis-metritis-agalaxia syndrome in sows, exudative epidermitis, and meningitis. However, off-label use for other conditions, such as blanket prophylactic treatments, has been reported (6). The summary of the product characteristics of Naxcel, a vegetable oil-based suspension of ceftiofur crystalline free acid, reports that approximately 60% and 15% of the dose is excreted in the urine and feces, respectively, within 10 days after administration. The impact of the use of ESCs on the selection and dissemination of ESC-resistant *Enterobacteriaceae* is debated (7–9), and very few data are available concerning the impact of these antimicrobials on the bacterial populations that make up pig microbiota and participate in their metabolism. Using pigs inoculated with ESC-resistant (ESCR) *E. coli* and housed under controlled conditions, the present study aimed at evaluating the impact of a long-acting form of ESC on gut microbiota composition and metabolism and intestinal *E. coli* ESC resistance. Culture, molecular, and biochemical methods were used to monitor the modifications in microbiota composition, the ESC susceptibility of *E. coli*, and the levels of short-chain fatty acids (SCFA).

MATERIALS AND METHODS

Preparation of the ESCR *E. coli* strain. We prepared the ESCR *E. coli* strain inoculated in pigs so as to increase the likelihood of bacterial colonization in the study pigs. Thus, *E. coli* was first isolated on MacConkey medium after obtaining samples from the feces of piglets from the specific-pathogen-free (SPF) experimental swine herd at the Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES) Ploufragan laboratory (France). After identification per PCR (10), the strains were tested for the presence of K85, K87, K88, K81, K82, and F6(987P) antigens (typical of pig-pathogenic *E. coli* strains) with antisera (Biovac, France), and antimicrobial susceptibility was determined by the disk diffusion assay and interpreted according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (11). One randomly chosen pansusceptible isolate, *E. coli* UB12/059-3, was then made resistant to rifampin by culture in Mueller-Hinton medium (MH) containing 250 mg/liter rifampin. The rifampin-resistant mutant was then used as a receptor for *in vitro* conjugation with ESCR *E. coli* strain 05-M63-1 from our strain collection. This

TABLE 1 Body weight gains

Room	Group	Description	Body wt gain (mean ± SD) (kg)			
			D0–D7	D0–D14	D0–D21	D0–D37
1A	NT	Noninoculated, nontreated	6.7 ± 1.0[a]	13.5 ± 1.27	20.2 ± 2.5	26.85 ± 3.1
1B	T	Ceftiofur treated on D0	6.8 ± 1.5	13.2 ± 3.0	18.1 ± 3.6	24.4 ± 4.5
1C	M63	*E. coli* M63 inoculated on D0	6.1 ± 1.0	12.8 ± 1.7	17.6 ± 2.0	23.65 ± 2.5
2C[b]			6.2 ± 1.5			
1D	M63-T	Ceftiofur treated and *E. coli* M63 inoculated on D0	6.8 ± 0.8	13.4 ± 1.4	17.5 ± 1.5	25.2 ± 2.0
2D[b]			7.5 ± 0.7			

[a] No significant differences in body weight gains between rooms or groups for the different periods ($P > 0.05$) were observed.
[b] Pigs in rooms 2C and 2D were included for the first week only.

ESCR *E. coli* strain originated from fecal material obtained from a healthy pig at the slaughterhouse. Conjugation was performed by mixing equal cultures of the recipient strain UB12/059-3 and the donor strain *E. coli* 05-M63-1 in MH medium containing rifampin (250 mg/liter) and cefotaxime (32 mg/liter). One ESCR transconjugant (M63) was obtained and further characterized by determining the MICs of different antimicrobials by microdilution using a Sensititre plate (Trek/Biocentric, Bandol, France), according to Clinical and Laboratory Standards Institute guidelines (12), identifying its phylogenetic group (13), and screening for the presence of the $bla_{CTX\text{-}M\text{-}1}$ gene (14). The transconjugant strain was tested for the presence of K85, K87, K88, K81, K82, and F6(987P) antigens. To check the *in vivo* fitness of the transconjugant strain, a preliminary experiment was performed and confirmed that the transconjugant strain could colonize pigs from the SPF herd and could be transmitted between pigs housed in the same pen (data not shown). The $bla_{CTX\text{-}M\text{-}1}$ plasmid contained in the transconjugant was sequenced using MiSeq Illumina technology (paired-end 2 × 250 nucleotides). Sequences were cleaned with the Trimmomatic 0.32 (15) software (ILLUMINACLIP:illumina_oligos_and_reverse_
complements:2:30:5:1:true LEADING:3 TRAILING:3 MAXINFO:40:0.2 MINLEN:36 options). Two Bowtie 2 (16) alignments were performed (-nondeterministic -very sensitive options) on cleaned sequences to the $bla_{CTX\text{-}M\text{-}1}$ gene (GenBank accession no. DQ915955), so as to evaluate sequencing depth, and to phiX17. This second alignment was made to remove reads matching to phiX174 material, which is used in Illumina sequencing in cases of very redundant samples. The unaligned reads were downsampled to fit a global coverage estimation of 80×. The remaining reads were provided to the SPAdes 3.1.1 *de novo* assembler (17). Redundant or poorly covered contigs were filtered out. The resulting assembly was submitted to ResFinder (18) to identify resistance genes and to the Rasta-Bacteria program version 2.12 (http://genoweb1.irisa.fr/duals/RASTA-Bacteria/) (19) to identify putative toxin-antitoxin systems.

Animals and experimental design. Experiments were performed in accordance with the animal welfare experimentation recommendations issued by the Direction Départementale de la Protection des Populations des Côtes d'Armor (ANSES registration no. B-22-745-1), and were approved by the ComEth ANSES/ENVA/UPEC ethics committee (authorization no. 12-003). Six animal rooms were used to house 48 Large White piglets obtained from the SPF herd at the ANSES Ploufragan laboratory. The piglets of 7 weeks of age were the progeny of five different sows and were randomized before the experiment. Each room contained eight piglets placed in two pens of four animals. The experimental groups were as follows: the nontreated (NT) control group (8 pigs) in room 1A, the ceftiofur-treated (T) group (8 pigs) in room 1B, the *E. coli* M63-inoculated group (M63) in rooms 1C (8 pigs) and 2C (8 pigs), and the *E. coli* M63-inoculated ceftiofur-treated group (M63-T) in rooms 1D (8 pigs) and 2D (8 pigs) (Table 1). The animals in rooms 1A to 1D were monitored up to 35 days after inoculation (D35), whereas the animals of rooms 2C and 2D were monitored up to D8 only, because they were then included in another study. The animals did not receive any treatment with any antibiotics prior to the assay. The same nonsupplemented starter diet was offered to the animals up to D2, and then pigs were fed with a nonsupplemented standard growing diet until the end of the experiment. These diets were formulated to cover the nutritional requirements of the pigs. Strict biosecurity measures were implemented to avoid contamination of the pigs, including the use of an air filtration system and airlocks for each room, the use of unit-specific clothes for each room, and compulsory showers after visiting the pigs.

E. coli M63 inoculations were performed on D0. Each piglet from groups M63 and M63-T was orally given a 10-ml suspension prepared from *E. coli* M63 cultivated on MH agar containing cefotaxime (2 mg/liter). The titer of the suspension was determined by spreading 10-fold dilutions on MH plates. Piglets from groups NT and T were similarly inoculated with sterile medium.

On D0, just after *E. coli* M63 inoculation, piglets from groups T and M63-T were given a single intramuscular injection of a vegetable oil-based suspension of ceftiofur crystalline free acid (Naxcel; Pfizer) at the recommended dose of 5 mg/kg of body weight. The weight of each animal was individually recorded once a week. During the week, daily clinical examinations consisted of looking for general clinical signs and taking rectal temperatures. The pigs were euthanized on days 35 to 37 postinoculation by intravenous injection of sodium pentobarbital, followed by exsanguination, and lesions were observed. Individual fecal samples were collected from all animals from the day before inoculation (D−1) and on D1, D3, D7, D10, D13, D17, D22, and D28. The fecal samples were immediately placed in generators for anaerobic bacteria (GENbag anaer; bioMérieux, Marcy l'Etoile, France), frozen in liquid nitrogen, and then stored at −70°C until analysis.

Bacteriological analysis. Bacteriological analyses were conducted on samples collected from the six animal rooms during the first week and samples from the four animal rooms (rooms 1A to 1D) thereafter. The titers of putative *E. coli* (red/pink colonies surrounded by hazy medium) for D−1, D1, D3, D10, and D22 and of ESCR *Enterobacteriaceae* at all sampling times of the individual fecal samples were determined by spreading 100 μl of 10-fold dilutions on MacConkey agar plates with or without 2 mg/liter cefotaxime in triplicate. After incubation, the colonies were enumerated, and the titers were calculated for each pig and each day. The detection limit was 100 CFU/g of feces. From the cefotaxime-supplemented plate, five isolates per pig per day were tested for susceptibility to cefotaxime and to rifampin by restreaking on MacConkey plates containing either 2 mg/liter cefotaxime or 250 mg/liter rifampin. All cefotaxime-resistant and rifampin-susceptible isolates and one cefotaxime- and rifampin-resistant isolate per group and per day were stored for further analysis. They were identified with *E. coli*-specific PCR (10, 20). The presence of the $bla_{CTX\text{-}M\text{-}1}$ gene was screened by PCR (14), and the phylogenetic groups of the *E. coli* isolates were determined (13). After digestion with SmaI, the pulsed-field gel electrophoresis (PFGE) profiles of a few isolates were compared to the profile of the inoculated *E. coli* M63 strain (21).

Molecular analysis. DNA extracts were prepared from 0.2 g of individual fecal samples, according to Yu and Morrison (22), followed by use of Qiagen's DNA stool kit (Qiagen, Courtaboeuf, France). Each DNA

extract was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Courtaboeuf, France) and was then adjusted to a concentration of 10 ng/μl. For all samples collected from pigs in rooms 1A to 1D, previously validated quantitative PCR (qPCR) analyses, all targeting 16S rRNA gene fragments, were carried out to evaluate the change in abundance of the total bacterial population and of the numbers of *Bacteroides/Prevotella*, *Bifidobacterium*, *E. coli*, *Enterococcus*, and *Lactobacillus/Leuconostoc/Pediococcus* (23). qPCR for quantification of $bla_{CTX\text{-}M\text{-}1}$ gene copies was performed (24) for samples collected from the six animal rooms during the first week and from rooms 1A to 1D thereafter; the copy numbers were determined by comparison with decimal dilutions of plasmid DNA prepared from the $bla_{CTX\text{-}M\text{-}1}$ gene previously cloned in the plasmid pCR4-TOPO in the One Shot TOP10 *E. coli* strain (Life Technologies, St. Aubin, France), according to the manufacturer's instructions.

Short-chain fatty acid assay. Short-chain fatty acids (SCFA), which are considered to be fermentation markers, were analyzed by gas chromatography, as described by Gérard-Champod et al. (25). Only samples collected from the four animal rooms 1A to 1D were analyzed. Acetic, propionic, isobutyric, butyric, isovaleric, valeric, and caprylic acids and heptanoate concentrations were determined. Assays were performed after a deproteinization step with phosphotungstic acid and using 2-ethylbutyric acid as an internal standard.

Statistical analysis. For measurement of body weight gains, culture results, and numbers of $bla_{CTX\text{-}M\text{-}1}$ copies, all 48 pigs in rooms 1 to 6 were included to study the effect of the ceftiofur treatment during the first week. For the following days, or for quantification of the bacterial groups by qPCR and metabolites, only animals from rooms 1A to 1D were included.

Differences between weight gains of animals or culturable *E. coli* titers from the different groups were analyzed using the Kruskal-Wallis test, followed by the Wilcoxon test. The individual titers of culturable *E. coli* and ESCR *Enterobacteriaceae* and gene copy numbers were $\log_{10}$ transformed, and significant differences between the M63 and M63-T groups were compared using the Wilcoxon test.

For data regarding the quantification of bacterial populations by qPCR and SCFA analysis, a nonparametric Mann-Whitney test was applied to ensure that the four groups were similar at the beginning of the experiment on D−1. After normalization [calculated with the formula $\Delta\log = \log(Dx) - \log(D-1)$], values were grouped per period (first week, D1, D3, and D7; second week, D10 and D13; third period, D17, D22, and D28), and the normalized data of the different groups for the different periods were compared using a nonparametric Mann-Whitney test.

Distributions were compared using the chi-square test or Fisher's exact test. For all tests, P values of <0.05 were considered statistically significant differences. All analyses were carried out in R 3.0.0 (R Core Team, 2013) using the stats package.

Nucleotide sequence accession numbers. The nucleotide sequences of the $bla_{CTX\text{-}M\text{-}1}$ plasmid of *E. coli* M63 were deposited under GenBank accession numbers KR494248, KR494249, KR494250, KR494251, and KR494252.

RESULTS

Preparation and characterization of the inoculated strain. *E. coli* UB12/059-3, isolated from our SPF pig herd, belonged to phylogenetic group B1, and the disk diffusion assay showed that it was susceptible to amoxicillin, amoxicillin-clavulanic acid, cephalothin, cefoxitin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, neomycin, nalidixic acid, and ciprofloxacin. After conjugation of the rifampin-resistant mutant of *E. coli* UB12/059-3 and ESCR *E. coli* 05-M63-1, one *E. coli* M63 isolate was obtained. Like the recipient *E. coli* strain UB12/059-3, *E. coli* M63 belonged to the B1 phylogenetic group and gave negative results with the tested K85, K87, K88, K81, K82, and F6(987P) antisera. The MICs of *E. coli* M63 are presented in Table 2. *E. coli* M63 was resistant to β-lactams, including ESC (cefotaxime), streptomycin, and trimethoprim-sulfamethoxazole. Sequencing of the entire plasmid of *E. coli* M63 showed that it contains the following resistance genes: $bla_{CTX\text{-}M\text{-}1}$, $bla_{CMY\text{-}59}$, *sul2*, *dfrA17*, and *aadA5*, and it is a sequence type 12 (ST-12) IncI1 plasmid (ardA_4, pilL1, repl1_1, sogS_4, and trbA_3 alleles). The plasmid maintenance protein, toxin CcdB and antitoxin CcdA protein, and toxin RelE and antitoxin RelB proteins were detected.

TABLE 2 MICs of antimicrobials on *E. coli* M63

Antimicrobial[a]	MIC (mg/liter)
AMP	>128
CTX	>2
FOX	≤8
MEM	≤0.12
CHL	8
COL	≤0.5
STR	32
GEN	0.5
TET	4
SMX	>512
SXT	>16/304
NAL	4
CIP	0.03

[a] AMP, ampicillin; CTX, cefotaxime; FOX, cefoxitin; MEM, meropenem; CHL, chloramphenicol; COL, colistin; STR, streptomycin; GEN, gentamicin; TET, tetracycline; SMX, sulfamethoxazole; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin.

Inoculation, clinical signs, and body weight gains. Piglets were inoculated with *E. coli* M63 (78×10^8 CFU per pig). Before the beginning of the trial, two piglets in room 2D showed symptoms of diarrhea, and the presence of rotavirus was detected by enzyme-linked immunosorbent assay (ELISA) at the local veterinary testing laboratory (data not shown). Subsequently, signs of diarrhea were observed between D17 and D21 in seven and three animals from rooms 1C and 1D, respectively, again with a confirmed presence of rotavirus. Otherwise, no diarrhea was detected during the 3 weeks following inoculation of *E. coli* M63. The rectal temperatures of the pigs remained at <40.0°C for the 2 weeks following inoculation, except on D1 (four animals from the M63-T group with temperatures of 40.1 to 40.3°C) and on D2 (one animal from the M63 group with temperature of 41.0°C). No other clinical signs or lesions were observed postmortem.

The mean body weight gains of the animals are presented in Table 1. There were no significant differences between the rooms or groups for any of the postinoculation periods.

Isolation of *E. coli* and ESCR *Enterobacteriaceae* in fecal samples. The titers of culturable *E. coli* obtained on D−1, D1, D3, D10, and D22 are given in Table 3. The day after injection of ceftiofur, the titer was significantly lower in the T group than that in all other groups ($P < 0.01$). On D3 and on D10, the titers of the two treated groups (T and M63-T groups) were significantly lower than those of the NT group ($P < 0.05$ for each comparison).

No ESCR *Enterobacteriaceae* were detected in fecal samples before *E. coli* M63 inoculation or in noninoculated animals during the whole assay period. For the inoculated pigs, individual titers were determined, and the group means are given in Table 4. Apart from the week following ceftiofur administration, individual fecal titers were usually between 10^3 and 10^5 CFU of ESCR *Enterobacteriaceae* per gram of fecal sample. However, one pig (no. 4314)

TABLE 3 Culturable *E. coli* titers in fecal samples

Group[a]	Culturable *E. coli* titer (mean $\log_{10}$ CFU/g ± SD) on[b]:				
	D−1	D1	D3	D10	D22
NT	6.15 ± 0.31^{1}	6.30 ± 0.51^{1}	6.46 ± 0.47^{1}	6.33 ± 0.49^{1}	6.35 ± 0.71^{1}
T	6.21 ± 0.45^{1}	5.32 ± 0.35^{2}	4.67 ± 0.43^{3}	5.83 ± 0.28^{345}	6.59 ± 0.42^{1}
M63	6.08 ± 0.65^{1}	6.13 ± 0.36^{1}	6.24 ± 0.39^{12}	5.94 ± 0.45^{14}	6.53 ± 0.39^{1}
M63-T	5.74 ± 0.32^{1}	6.27 ± 0.47^{1}	5.67 ± 0.62^{2}	5.58 ± 0.34^{25}	6.26 ± 0.37^{1}

[a] Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T.
[b] Values in a given row with different superscript numbers are significantly different ($P < 0.05$). The means were calculated from the data of eight pigs per group, except on D−1, D1, and D3, for which the M63 and M63-T groups included 16 pigs each.

from the M63 group regularly excreted more ESCR *Enterobacteriaceae* than the other pigs in the same group. The ESCR *Enterobacteriaceae* titers in the fecal samples collected from this pig on D3, D7, D10, and D13 were 1.3 to 2.1 $\log_{10}$ higher than those of all but one sample collected from the other pigs, and on D17, its titer was 0.6 $\log_{10}$ higher than that for all other animals. Thus, comparisons of the titers of the M63 and M63-T groups were performed either with data from all pigs or after excluding this high-shedding pig. In both cases, significant differences ($P < 0.02$) between the T and NT groups were observed on D1, D3, and D28, with titers from treated animals exceeding those from nontreated ones. No significant differences were observed on the other days. Furthermore, given that the presence of a high shedder in the pen may have enhanced the recontamination of the other animals of the same pen, the statistical analysis was also performed after excluding this pen from the data set and again showed that there were significant differences on D1 ($P < 0.05$), D3 ($P < 0.01$), and D28 ($P < 0.05$).

Characterization of ESCR *E. coli*. A total of 759 isolates obtained on cefotaxime-supplemented medium from the M63 and M63-T groups were tested for cefotaxime and rifampin resistance. The results confirmed that all isolates were resistant to cefotaxime. During the first days after inoculation, all isolates were also resistant to rifampin, like the *E. coli* M63 strain used for inoculation (Table 5). However, thereafter, the mean percentages of rifampin-resistant isolates decreased to a minimum of 47.5% on D28 in the M63 group and 70% on D17 in the M63-T group. Interestingly, the individual ratios of rifampin-resistant and rifampin-susceptible isolates varied widely, with some pigs yielding only rifampin-resistant isolates during the whole experiment, some other pigs (such as the high-shedding pig) yielding only rifampin-resistant isolates except on D28, when all isolates were rifampin susceptible, and pigs for which susceptibility varied from one sampling to another.

One hundred eleven isolates, including all 89 rifampin-susceptible and 22 rifampin-resistant isolates (at least one per pen per day), were analyzed further. All belonged to the *E. coli* species, but 15 isolates collected on D22 and D28 in the M63 and M63-T groups did not show characteristic *E. coli* colonies on MacConkey agar; these isolates gave typical blue colonies on tryptone bile X-glucuronide (TBX) medium (data not shown), a characteristic of beta-glucuronidase-positive strains, but apparently lacked the *uidA* sequences, a target of the PCR described by Bej et al. (20). However, these isolates were positive for the PCR based on the 16S rRNA gene (10) and were identified as *E. coli* by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (data not shown). All of the 111 cefotaxime-resistant isolates harbored the $bla_{\text{CTX-M-1}}$ gene. All of the 22 cefotaxime- and rifampin-resistant isolates tested that were obtained from D1 to D28 belonged to the same B1 phylogenetic group as the *E. coli* M63 strain. In the M63 group, the identified phylogroups of 50 cefotaxime-resistant rifampin-susceptible isolates were B1 (30 isolates), A/C (4 isolates), D/E (11 isolates), F (1 isolate), and E/clade I (4 isolates). In the M63-T group, the phylogroups of the cefotaxime-resistant rifampin-susceptible isolates included B1 (25 isolates), D/E (5 isolates), B2 (3 isolates), and F (6 isolates). The three B2 isolates were obtained from two animals from the same pen. D/E isolates were obtained from four animals housed in the same pen of room 1C but from only one pig from room 1D. All isolates of the F group were obtained from two pigs of the same pen in room 1D.

The PFGE analysis of 14 isolates showed that one rifampin-resistant isolate of B1 group showed the profile of *E. coli* M63, while another one from the B1 group, which was rifampin susceptible, had a profile distinct from that of *E. coli* M63. Two isolates that had been obtained from two different pigs from the same pen of M63 group on D22 and D28, were assigned to the D/E group, and were negative for the *uid* gene had the same PFGE profile. Two isolates obtained from two different pigs of the same pen from the M63 group on D28, belonging to the E/clade I group, showed the same PFGE profile. A total of 8 different profiles were observed for the 13 rifampin-susceptible isolates tested (data not shown).

TABLE 4 Cefotaxamine-resistant *Enterobacteriaceae* (CRE) in the individual fecal samples

Group[a]	CRE titer (mean $\log_{10}$ CFU/g ± SD) on[b]:							
	D1	D3	D7	D10	D13	D17	D22	D28
M63	4.7 ± 2.4^{1}	3.7 ± 1.7^{1}	3.3 ± 2.0^{1}	4.2 ± 1.1^{1}	4.7 ± 1.8^{1}	4.5 ± 1.7^{1}	4.3 ± 1.4^{1}	2.8 ± 1.2^{1}
M63-T	5.8 ± 1.7^{2}	5.25 ± 0.95^{2}	3.35 ± 1.35^{1}	3.8 ± 0.4^{1}	3.7 ± 0.4^{1}	3.1 ± 0.7^{1}	4.0 ± 0.5^{1}	3.9 ± 0.6^{2}

[a] No cefotaxime-resistant *Enterobacteriaceae* were isolated from the NT and T groups or before inoculation of *E. coli* M63. Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T.
[b] On D1, D3, and D7, 16 samples per group were tested; on D10, D13, D17, D22 and D28, eight samples per group were tested. Values in a row with different superscript numbers are significantly different ($P < 0.05$).

TABLE 5 Ratios of rifampin-resistant isolates among cefotaxime-resistant *E. coli* isolates

Group[a]	No. of rifampin-resistant isolates/no. of cefotaxime-resistant *E. coli* isolates (%) on[b]:							
	D1	D3	D7	D10	D13	D17	D22	D28
M63	65/65[1] (100)	51/51[1] (100)	54/57[1] (95)	36/40[1] (90)	26/35[1] (74)	38/40[1] (95)	29/40[1] (72.5)	19/40[1] (47.5)
M63-T	79/79[1] (100)	76/76[1] (100)	36/36[1] (100)	36/40[1] (90)	35/40[1] (87.5)	28/40[2] (70)	29/40[1] (72.5)	33/40[2] (82.5)

[a] Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T.
[b] Values in a row with different superscript numbers are significantly different ($P < 0.05$).

Quantification of $bla_{CTX\text{-}M\text{-}1}$ by qPCR. The detection limit of the $bla_{CTX\text{-}M\text{-}1}$ PCR was 10^4 copies per assay. The results for $bla_{CTX\text{-}M\text{-}1}$ quantification are given in Table 6. All samples collected before inoculation or from noninoculated pigs were negative. The total numbers of samples testing positive from D1 to D28 according to qPCR were 34/85 (40%) for M63 pigs versus 31/86 (36%) for M63-T ones ($P > 0.05$, chi-square test). During the week following the ceftiofur injection, the numbers of positive samples in the M63 and in the M63-T groups were 19/47 (40%) and 28/48 (58%), respectively ($P > 0.05$, chi-square test), but considering samples collected after the first week, 15/39 (38.5%) samples were found positive in M63 pigs, compared with only 3/38 (8%) in M63-T pigs ($P = 0.002$, Fisher's exact test). Interestingly, all 15 positive samples from the NT group were obtained from the pen housing the high-shedding pig, and all pigs from this pen tested from D10 to D22 were always positive. Considering either the 16 pigs per group during the first week or after exclusion of the high-shedding pig, the number of copies of the $bla_{CTX\text{-}M\text{-}1}$ gene in M63-T pigs was significantly higher than that in the M63 group on D1 ($P < 0.01$, Wilcoxon test) and D3 ($P < 0.001$). In contrast, M63-T animals excreted fewer copies of the $bla_{CTX\text{-}M\text{-}1}$ gene on D13 ($P = 0.03$, Wilcoxon test, considering all pigs, or $P = 0.057$ after exclusion of pig no. 4314).

Composition of the fecal microbiota. On D−1, for most bacterial groups, the composition of the fecal microbiota based on molecular methods was similar in the four treatment groups ($P > 0.05$), with initial mean ± SD titers of 11.21 ± 0.15 $\log_{10}$ copies of 16S rRNA gene for all bacteria, 7.98 ± 0.50 $\log_{10}$ for *E. coli*, 7.69 ± 0.71 $\log_{10}$ for *Bifidobacterium*, 9.45 ± 0.15 $\log_{10}$ for *Bacteroides/Prevotella*, and 9.25 ± 0.57 $\log_{10}$ for *Lactobacillus/Leuconostoc/Pediococcus*. The mean ± SD copy number for *Enterococcus* was 7.19 ± 0.38 $\log_{10}$, but there were differences between groups ($P < 0.05$ for each comparison). However, the means for *Enterococcus* of the four groups for the first and second weeks were very similar, ranging from 7.02 to 7.13 and 7.21 to 7.47 $\log_{10}$, respectively; regarding the trends over the whole period (Fig. 1), the increase in enterococci was significantly more pronounced in the M63-T group than in all other groups ($P < 0.001$ for each comparison). Another significant difference on D−1 was detected between the NT and M63-T groups for *Bifidobacterium* ($P < 0.05$). Afterwards, the *Bifidobacterium* counts decreased in the NT group but increased in all the other groups during the first week ($P < 0.01$ for each comparison) and the whole period ($P < 0.01$ for each comparison).

In the NT group, the mean variations of the bacterial groups were <1 $\log_{10}$ during the different periods, except for the *Lactobacillus/Leuconostoc/Pediococcus* population, which showed a pronounced decrease during the second (−1.4 $\log_{10}$) and third periods (−1.7 $\log_{10}$) compared to D−1 (Fig. 1). During the whole period, this decrease was also recorded for the other groups but was significantly less pronounced in the M63 and M63-T groups ($P < 0.001$).

During the first week after treatment, the main significant difference between groups was the drop in the *E. coli* 16S rRNA gene copy numbers in the T group (−1.6 $\log_{10}$ compared to that at D−1) compared with the NT group (−0.4 $\log_{10}$ compared to that at D−1, $P < 0.001$). However, at the end of the experiment, the mean values for the four groups were very close, ranging from 7.94 (M63-T group) to 8.14 $\log_{10}$ (T group).

We paid particular attention to the results obtained for the high-shedding pig (pig no. 4314). Interestingly, on D−1, compared with the 31 other tested animals, according to qPCR, this pig

TABLE 6 Number of $\log_{10}$ copies of the $bla_{CTX\text{-}M\text{-}1}$ gene and numbers of pigs shedding the $bla_{CTX\text{-}M\text{-}1}$ gene, as determined by qPCR

Group[a]	Results on[b]:							
	D1	D3	D7	D10	D13	D17	D22	D28
M63								
$\log_{10}$ copies/10 ng of DNA (mean ± SD)	2.87 ± 1.50[1c]	0.73 ± 1.34[1]	0.51 ± 1.45[1]	1.49 ± 1.63 [1]	1.86 ± 2.18 [1]	1.61 ± 1.81 [1]	0.93 ± 1.16 [1]	0.00 ± 0.00 [1]
No. of positive samples/no. of tested samples	13/16	4/15	2/16	4/8	4/8	4/8	3/7	0/8
M63-T								
$\log_{10}$ copies/10 ng of DNA (mean ± SD)	3.76 ± 1.55[2]	2.35 ± 1.57[2]	0.29 ± 0.20[1]	0.25[1] ± 0.71 [1]	0.00 ± 0.00 [2]	1.11[1] ± 1.72 [1]	0.00 ± 0.00 [1]	0.00 ± 0.00 [1]
No. of positive samples/no. of tested samples	14/16	12/16	2/16	1/8	0/8	2/6	0/8	0/8

[a] Results for samples from NT and T animals or collected before inoculation were negative. Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T.
[b] Values in a row with different superscript numbers are significantly different ($P < 0.05$).
[c] Results include the high-shedder pig.

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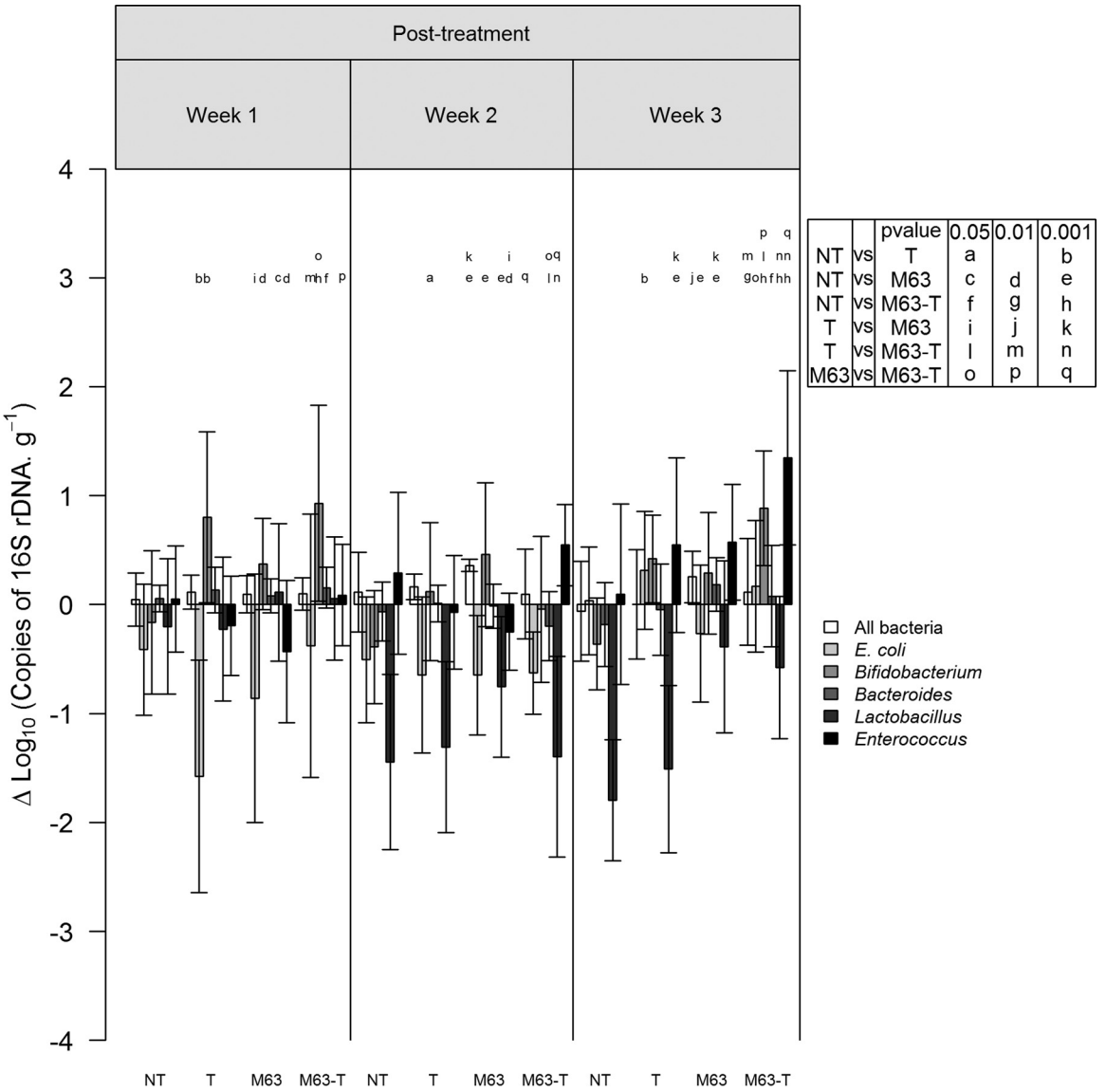

FIG 1 Changes in the main bacterial populations of the fecal microbiota in the different treatment groups. After normalization [Δlog = log(Dx) − log(D−1)], values were grouped per period (first week: D1, D3, and D7; second week: D10 and D13; third period: D17, D22, and D28). Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T. Means and standard deviations are shown.

had the highest *E. coli* level (9.00 $\log_{10}$) and the lowest *Bacteroides* level (9.02 $\log_{10}$). On six of the eight other sampling times after inoculation, this pig had the highest level for *E. coli* 16S rRNA gene copies in its group.

Short-chain fatty acids. At the beginning of the experiment, the mean ± SD levels of the major SCFAs (acetate, 82.38 ± 12.27 μmol/g), propionate (25.07 ± 4.53 μmol/g), and butyrate (30.28 ± 8.13 μmol/g) were similar between the groups ($P >$ 0.05). For minor SCFAs, the mean ± SD levels on D1 were much lower: 1.81 ± 0.78 μmol/g for isobutyrate, 2.70 ± 1.29 μmol/g for isovalerate, 0.01 ± 0.03 μmol/g for heptanoate, 2.60 ± 0.86 μmol/g for valerate, and 0.47 ± 0.65 μmol/g for caproate; significant differences were observed for isobutyrate and isovalerate in the NT group compared with the M63 groups ($P < 0.05$ for each), heptanoate in the T group compared with the M63-T group ($P <$ 0.001), and valerate and caproate in the M63 group compared with the M63-T group ($P < 0.05$ for each). Thus, for the following periods, only changes in the main SCFAs are described (Fig. 2). During the first, second, and third periods, acetate levels increased in the NT group but decreased in the other groups ($P <$ 0.02). On the first week after inoculation, the butyrate levels remained relatively stable in the NT group, a pattern that was significantly different from the decrease observed in the two M63-inoculated groups ($P < 0.001$), and the difference persisted during the second week ($P < 0.02$); similarly, during the first week, the decrease in the acetate levels in the T group was low compared with the decrease observed in the two *E. coli* M63-inoculated groups ($P < 0.02$). For propionate, the main difference was between the relative stability observed in the NT group and the decrease in the M63-T group during the first and second weeks ($P < 0.001$ for each pair) and between the M63-T group and the M63 group or the T group during the first and

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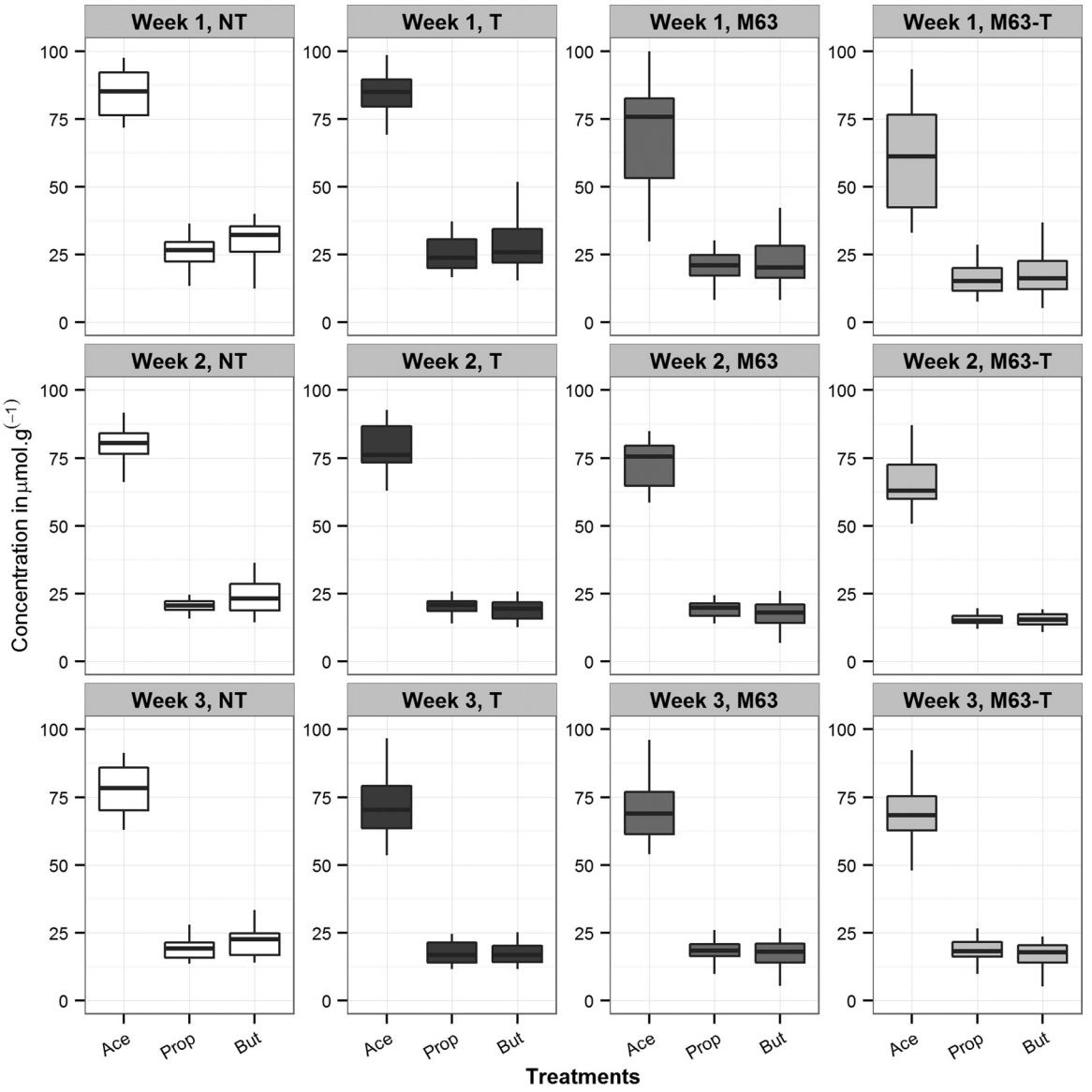

FIG 2 Change in short-chain fatty acid content of the fecal microbiota in the different treatment groups. Ceftiofur was given on D0 to groups T and M63-T, and *E. coli* M63 was inoculated on D0 in groups M63 and M63-T. The values are grouped per period (week 1: D1, D3, and D7; week 2: D10 and D13; during and after week 3 period: D17, D22, and D28. The black band in each box is the median, the bottom and top of each box indicate the 25th and 75th quartiles, respectively, and the whiskers show the standard deviations. Ace, acetic acid; Prop, propionic acid; But, butyric acid.

second weeks ($P < 0.05$ for each pair), but the propionate means were very similar during the last period (from 17.64 µmol/g in the T group to 19.56 µmol/g in the NT group).

DISCUSSION

The experimental model described here was based on the preparation of ESCR *E. coli*, obtained by conjugating an isolate from our SPF swine herd to a $bla_{CTX\text{-}M\text{-}1}$-harboring *E. coli* strain, because this gene codes for the most frequently described extended-spectrum β-lactamase in pigs in Europe. The resulting *E. coli* M63 strain belonged to the same phylogenetic group as the recipient and showed β-lactam resistance, including resistance to ESCs, streptomycin, and trimethoprim-sulfamethoxazole, according to the resistance genes carried on the plasmid.

The inoculation of *E. coli* M63 resulted in limited symptoms in a few animals. The strain appeared to persist in all the inoculated pens up to the end of the experiment, as many isolates were rifampin and cefotaxime resistant and belonged to the *E. coli* M63 phylogenetic group. It is also noteworthy that even in nontreated pigs, *in vivo* transfer of the ESC resistance between different *E. coli* strains occurred and underlines the dissemination capacity of this resistance.

According to qPCR, the bacterial composition of the SPF pig microbiota was similar to that of other farm animals (10). In the different treatment groups, the *Lactobacillus/Leuconostoc/Pediococcus* population decreased over time, as previously reported, and was related to the change in pig microbiota during the weeks following weaning (26). The main impact of the ceftiofur treatment

was the significant decrease in the *E. coli* population during the first days after injection, and this was confirmed by the culture results obtained on D1 to D10. However, on D22, the culturable *E. coli* levels were no longer different between the groups, and the numbers of 16S rRNA genes were slightly higher than their initial level. Thus, under our conditions, a single intramuscular injection of the long-acting form of ceftiofur at the recommended dose of 5 mg/kg of body weight led to a significant but transitory decrease in the *E. coli* population. Singer et al. (27) reported a similar pattern in dairy cattle after the use of ceftiofur (2.2 mg/kg, intramuscularly, once daily for 5 days), whereas for Cavaco et al. (28), administration of ceftiofur (3 mg/kg) once a day for 3 days by intramuscular injection did not result in significant differences in the average counts of total coliforms. The differences in dosage and pharmaceutical forms are probably partly responsible for such different results, and it would be interesting to determine the levels of ceftiofur in the intestinal tract on the days following the administration of the different presentations of ceftiofur.

Other significant changes in the bacterial populations were sometimes encountered. Thus, a minor difference (<1 $\log_{10}$) was observed for the *Bifidobacterium* taxon; this difference may result from the ceftiofur treatment, the *E. coli* M63 inoculation, or even the disturbance induced by the accidental *Rotavirus* contamination that occurred in the M63 and M63-T groups during the third and fourth weeks of the experiment. Few studies have reported the impact of cephalosporins on microflora. In a mouse intestinal colonization model, cefotaxime, given subcutaneously once a day for 3 days, has no impact on *Bacteroides* or aerobic Gram-positive flora evaluated using culture methods (29).

Concerning the main SCFAs, which are the products of the microbial breakdown of carbohydrates, provide energy to colonocytes, and stimulate sodium and water absorption in the colon (30), the main finding was the different patterns of acetate and butyrate changes in the NT group compared with the other groups, with higher levels of these SCFAs in the NT group. Thymann et al. (31) showed that that controlling weaning diarrhea with amoxicillin and zinc oxide reduces the concentration of SCFAs, an effect that is attributed to the general bacterial inhibitory effect and specific inhibition of certain tissue-associated pathogens. However, their experimental conditions were quite different, with the control group suffering from *E. coli* diarrhea.

Culture and nonculture methods were used to evaluate the impact of the ESC administration on the antimicrobial resistance of *Enterobacteriaceae*. Bacteriological methods made it possible to detect the transfer of the $bla_{\text{CTX-M-1}}$ gene from *E. coli* M63 to other *E. coli* strains. In both inoculated groups, this transfer occurred mainly after the first week after inoculation, as indicated by the finding of rifampin-susceptible ESCR *E. coli*. Transfer to isolates of the phylogenetic group B1 seemed the most frequent, occurring in 28/41 (68%) and 25/39 (64%) isolates among the cefotaxime-resistant and rifampin-susceptible isolates in the nontreated and in the treated animals, respectively. We did not attempt to study the diversity of these B1 *E. coli* isolates more thoroughly. Isolates of the B2, I/E, and F groups were each obtained from animals in only one pen, suggesting a single plasmid transfer event, followed by diffusion of the newly ESC-resistant isolate to other animals in the same pen. Transfer seemed limited to *E. coli*, as all the colonies, even the ones with noncharacteristic morphology on cefotaxime-supplemented MacConkey plates, could be identified as *E. coli* by a PCR test based on a 16S rRNA gene sequence and MALDI-TOF MS assay.

According to cultures on cefotaxime-supplemented medium, $>$10-fold higher titers were observed for treated animals 1 and 3 days after a single administration of the long-acting form of ceftiofur. The results of qPCR for the $bla_{\text{CTX-M-1}}$ gene also showed significantly higher numbers for the treated pigs on D1 and D3. Thereafter, no difference was detected by culture, except on the last sampling on D28, when again, the treated pigs excreted $>$10 times more resistant isolates than the nontreated pigs. Because this sampling was the last one, it is difficult to determine whether the increase observed on D28 would have continued over time. It is also important to underline the fact that one pig from the nontreated group excreted more ESC-resistant bacteria and $bla_{\text{CTX-M-1}}$ copies than the other pigs. Such differences in the excretion of resistant strains among animals has already been described (32), with the presence of high-density shedders ($>1 \times 10^4$ CFU of CTX-M-positive *E. coli*/g of feces) among cattle, chickens, and pigs. These high levels of CTX-M-positive *E. coli* shedding have been attributed to the use of cephalosporins or other coselecting antimicrobials. However, in our experiment, the high-shedding pig clearly belonged to the nontreated group, and this animal had never received antimicrobials. Interestingly, the fecal microbiota of this pig was already peculiar at the beginning of the experiment, showing the highest and lowest levels of *E. coli* and *Bacteroides*, respectively. This pig was also the lightest male piglet of its group at the time of inoculation. Super-shedding animals are probably quite important from an epidemiological point of view, because they can very effectively disseminate resistant bacteria, particularly in animals, such as pigs, with coprophagic or burrowing habits, and are probably a major source of contamination of carcasses in the food chain. In our experiment, the presence of one high shedder in the nontreated group probably had a direct effect on the ESCR *E. coli* mean of this group, and we tried to evaluate this impact by excluding this animal from the data set. Nevertheless, the difference between the groups was still significant for the same days (D1, D3, and D28 for culture and D1 and D3 for qPCR for $bla_{\text{CTX-M-1}}$). However, the exclusion of this animal does not eliminate the indirect effect of the high-level excretion of this pig, which might heavily recontaminate the other animals of the same pen and partially mask the impact of ceftiofur injection in the treated group. Thus, statistical analysis of the culture data was also performed excluding data from the pen with the high shedder; even in this case, the culture results were still significantly different for D1, D3, and D28 only.

In one study, the titers of CTX-M-producing *E. coli* of either indigenous or inoculated origin were about 100-fold higher in the feces of cephalosporin-treated pigs than in controls for $\geq$22 days after the end of treatment (28). The cephalosporins used in this study were either ceftiofur hydrochloride at 3 mg/kg or cefquinome at 2 mg/kg, both given once a day for 3 days. The apparently higher impact of these treatments compared to those in our study may be related to the differences in pharmacokinetics of the drugs or to differences in resistance levels, the fitness of the strains present in the digestive tract, the presence of a high-shedder in our nontreated experimental group, or other experimental conditions.

Epidemiological studies have also been conducted to decipher the relationships between ESC use and the prevalence of ESC resistance in pigs. A significant association between use and suscep-

tibility to cefotaxime has been reported for on-farm samples from piglets and slaughter pigs (7) and slaughterhouse samples in Denmark (33).Thus, to stop the increasing prevalence of ESCR *E. coli* in pigs, countries, such as Denmark, the Netherlands, and France, have decided on a voluntary ban on ESC in this livestock sector. This measure has already resulted in reduced ESC *E. coli* levels in slaughter pigs in Denmark (34) and the Netherlands (35) and in pathogenic *E. coli* from pigs in France (36), and it should be considered a promising approach for use in other countries. Importantly, our results also underline the fact that measures preventing the introduction of animals harboring ESCR *E. coli* and biosecurity measures are the best options for controlling ESC resistance.

Based on culture and molecular methods performed on fecal samples, the results of our study clearly show that in ESCR-contaminated animals, ceftiofur administration resulted in limited disturbance to pig microbiota, except for a significant, albeit transient, decrease in the culturable *E. coli* population, decreasing the levels of acetate and butyrate, but, more importantly, in a significant increase in the ESCR *E. coli* population. In treated and nontreated animals, the $bla_{\text{CTX-M-1}}$ gene was transferred to several strains of *E. coli*, confirming the high dissemination capacity of such resistance genes. Our observations also suggested that the presence of an ESCR *E. coli* high-shedding pig resulted in more frequent recontamination between pigs in the nontreated group, and such high shedders are probably of major importance in the epidemiology of resistance. Thus, limiting the use of ESCs, implementing strict biosecurity measures, checking the ESCR *Enterobacteriaceae* status of animals before their introduction on the premises, and/or detection of ESCR *E. coli* high-shedding animals are all important measures for preventing the introduction or limiting the selection for and spread of ESCR *Enterobacteriaceae*.

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