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Impact of two different colistin dosing strategies on healthy piglet fecal microbiota

M.A. Fleury [a,b,c], E. Jouy [a,b], F. Eono [a,b], R. Cariolet [a,b], W. Couet [d,e,f], P. Gobin [f], O. Le Goff [c], S. Blanquet-Diot [c], M. Alric [c], I. Kempf [a,b,*]

[a] ANSES, Laboratoire de Ploufragan-Plouzané, Ploufragan, France
[b] Université Bretagne, Loire, France
[c] Clermont Université, Université d'Auvergne, Centre de Recherche en Nutrition Humaine Auvergne, EA 4678 CIDAM, Conception, Ingénierie et Développement de l'Aliment et du Médicament, Clermont-Ferrand, France
[d] Inserm U1070, Pôle Biologie Santé, Poitiers, France
[e] Université de Poitiers, UFR Médecine-Pharmacie, Poitiers, France
[f] CHU Poitiers, Service de Toxicologie-Pharmacocinétique, Poitiers, France

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ABSTRACT

Colistin is often used in piglets but underdosing and overdosing are frequent. The impact of such administrations on fecal microbiota was studied. Piglets were given either underdoses of colistin by oral gavage for five days or overdoses by in-feed medication for 14 days. The composition of fecal microbiota was studied by quantitative PCR, 16S rRNA sequencing, culture of *Enterobacteriaceae*, and quantification of short-chain fatty acids (SCFAs). The mean colistin concentrations during the treatment for underdosed and overdosed groups were 14.4 μg/g and 64.9 μg/g of feces respectively. Whatever the piglet and the sampling day, the two main phyla were Firmicutes and Bacteroidetes, The main families were *Lactobacillaceae*, *Clostridiales*, *Lachnospiraceae* and *Ruminococcaceae*. The main perturbation was the significant but transitory decrease in the *Escherichia coli* population during treatment, yet all the *E. coli* isolates were susceptible to colistin. Moreover, colistin did not affect the production of SCFAs. These results show that under- or overdoses of colistin do not result in any major disturbance of piglet fecal microbiota and rarely select for chromosomal resistance in the dominant *E. coli* population.

1. Introduction

During the post-weaning period, piglets are submitted to a number of stress factors such as separation from the sow, mixing of litters, change of feed and infections caused mainly by pathogenic *Escherichia coli* and Rotavirus. Post-weaning diarrhea is one of the most frequently encountered diseases and results in animal suffering and huge economic losses. To control *E. coli* diarrhea, antimicrobials are often administered to the piglets. Colistin, belonging to the polymyxin family, is the most frequently used, as reported in several European studies (Callens et al., 2012; Casal et al., 2007). Colistin is a cationic agent which binds to the anionic bacterial outer membrane, leading to the disruption of bacterial integrity. It is active against most *E. coli* isolates, in addition to *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas aeruginosa*, and *Acinetobacter* spp. (Landman et al., 2008). There is very little or no absorption of colistin from the gastrointestinal tract of young pigs (Guyonnet et al., 2010). The dosage recommended by the summary of product characteristics (SmPC) for the treatment of gastrointestinal infections is 100,000 IU/kg of body weight (BW)/day for five days. However, in the field, colistin is often supplied as in-feed medication, with an incorporation dose of 3600 IU/g of feed, resulting—depending on the feed intake of animals—in a much higher dose of up to 170,000–200,000 IU/kg BW/day for 14 days. Inversely, it is also frequent to underdose either in the case of individual oral forced feeding with vomiting or oral administration problems (Hemonic et al., 2013) or in the case of in feed medication (Callens et al., 2012).

In human medicine, nephrotoxicity and neurotoxicity have led to the discontinuation of the routine use of colistin, but the emergence of multiresistant Gram negative bacteria has given rise to a renewed interest in the therapeutic properties of colistin (Landman et al., 2008). It should be noted that in humans, colistin is mainly administered intravenously or by nebulization as colistin methanesulfonate (CMS), a negatively charged prodrug with no antimicrobial activity. The oral administration of colistin sulfate for selective decontamination of the digestive tract in immunocompromised patients is limited to few countries and rather controversial because of the risk of resistance selection (Halaby et al., 2013).

It is now feared that the use of colistin in animals might select colistin-resistant bacteria which could then spread to humans and the

* Corresponding author at: ANSES, Laboratoire de Ploufragan, Unité Mycoplasmologie Bactériologie, 22440 Ploufragan, France.
E-mail address: isabelle.kempf@anses.fr (I. Kempf).

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European Medicines Agency (EMA) has recommended using colistin "for treatment (cure or metaphylaxis) of disease, and not for prophylactic use" (EUROPEAN-MEDICINES-AGENCY, 2013). Recent publications have revealed the emergence of plasmid-encoded resistance to colistin first in China (Liu et al., 2016) and then in other countries. These resistant bacteria were isolated from both human and animal sources (Olaitan et al., 2016; Perrin-Guyomard et al., 2016), so the use of colistin in animals will need to be re-assessed in the future. In the meanwhile, as with other antimicrobials, the use of this bactericidal agent may significantly perturb pig gut microbiota (Looft et al., 2012). The purpose of our study was thus to evaluate, through an animal test, the impact of a low dose (underdosing) or the field dose (overdosing) of colistin on the composition and fermentative activity of the fecal microbiota of pigs and on the resistance of intestinal *E. coli*.

2. Materials and methods

2.1. Animals, housing and experimental treatment

The experiment was conducted at ANSES Ploufragan, North-West France. Three independent air-filtered rooms were used to accommodate 24 Large-White piglets (8-week-old piglets weighing 16.2 ± 1.9 kg on average at the beginning of the experiment) from the ANSES Ploufragan specific-pathogen-free (SPF) herd, meaning that animals are free of the main viral and bacterial pathological agents. The animals were weaned four weeks before the experiment. The piglets were randomly assigned to three groups, taking into account litter origin, weight and sex: eight non-treated (NT) piglets in room 1; eight piglets in room 2 receiving colistin (Colivet solution, 2,000,000 IU/mL, CEVA, Libourne, France) by oral gavage at a low dose (50,000 IU/kg of body weight from Day 1 (D1) to D5 (colistin low dose group (CL))) and eight piglets in room 3 receiving an in-feed colistin medication (Concentrat VO 49-2 Colistine 200 SOGEVAL, Laval, France) at a dose of 3600 IU/g of feed (colistin high dose group (CH)). The commercial medicated feed was given ad libitum from day D1 to D14, and the same non-supplemented commercial feed was offered to the other two animal groups. In each room, the pigs were housed in two pens on flat decks. Daily clinical examinations consisted of recording clinical signs and rectal temperatures. Individual body weight was also recorded each week during the experiment and feed intake was measured in each pen. The experiment was terminated four weeks after the beginning of the treatment by euthanasia by intravenous injection of sodium pentobarbital followed by exsanguination and necropsy of four animals from each group, the remaining piglets being raised until slaughter. The experiment was performed in accordance with French animal welfare regulations and the protocol was approved by the ComEth ANSES/ENVA/UPEC ethical committee (authorization 14/02/12-3).

2.2. Sampling

Fecal samples were collected from each pig two or three times a week. Because all the animals could not be sampled on the same day, pigs in the NT group were sampled on D2, D10, D15 and D24; those in the CL group on D0, D2, D4, D7, D11 and D18 and those in the CH group on D0, D3, D9, D14, D18 and D22. Samples were immediately placed in generators for anaerobic bacteria (GENbag anaer, Biomerieux, Marcy l'Etoile, France) and stored at −70 °C until analysis.

2.3. Bacteriological examinations

For each individual fecal sample, ten-fold dilutions were prepared and 100 μL inoculated on McConkey agar medium (Oxoid, Basingstoke, England) to enumerate presumptive lactose-positive *E. coli* (red/pink colonies surrounded by a hazy medium). As far as possible, one randomly chosen typical *E. coli* colony per pig and per day was restreaked on Mueller Hinton medium (Becton Dickinson, Le Pont de Claix, France) and stored for further analysis. After identification by PCR (Furet et al., 2009), a standardized inoculum of each *E. coli* isolate was deposited on Mueller Hinton agar containing colistin sulfate (2 mg/L, Sigma, Saint-Quentin Fallavier, France) according to the CLSI method (CLSI, 2008) for determining Minimum Inhibitory Concentration (MIC). According to the EUCAST epidemiological cut-off (http://mic.eucast.org), the isolates with an MIC of colistin higher than 2 mg/L were classified as resistant.

2.4. Bacterial quantification by real-time PCR

DNA extracts were prepared from 0.2 g of individual fecal samples using the previously described protocol (Yu and Morrison, 2004) followed by Qiagen's DNA stool kit (Qiagen, Courtaboeuf, France). The same protocol was used to prepare DNA from bacterial cultures. DNA extracts were stored at −20 °C until analysis. Each DNA extract was quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Courtaboeuf, France) and was then adjusted to a concentration of 10 ng/μL.

Previously validated quantitative PCR (qPCR) analyses (Saint-Cyr et al., 2014), all targeting 16S rRNA gene fragments (Furet et al., 2009; Saint-Cyr et al., 2013), were carried out to assess changes in the abundance of the total bacterial population and of major bacterial groups characterizing the swine gut microbiota: *Bacteroides*/*Prevotella*, *Bifidobacterium*, *E. coli*, *Enterococcus* and *Lactobacillus*/*Leuconostoc*/*Pediococcus* according to previously published methods (Table 1). Briefly, PCR reactions were performed in a final volume of 10 μL containing 1 μL of diluted DNA sample, 0.2 μM of each primer, 0.25 μM of TaqMan® probe and 1× of IQ Supermix (Bio-Rad, Marnes la Coquette, France) or IQ SYBR-Green (Bio-Rad). The *E. coli* qPCR used a concentration of 0.3 μM of each primer and 0.1 μM of probe. The amplification program was composed of an initial denaturation of 95 °C for 10 min., 40 cycles of 95 °C for 30 s and 60 °C for 60 s, and a final melting-curve for SYBR-Green tests. For each sample and each bacterial group, results were expressed in $\log_{10}$ copies of 16S rRNA genes per g of fecal material.

2.5. Microbiota analysis by 16S rRNA gene sequencing

Three piglets (named A, B and C) from the CH group were randomly chosen for 16S rRNA sequencing analyses. Total DNA quantity and quality were prepared following MR DNA recommendations (www.mrdnalab.com, Shallowater, TX, USA) and 16S rDNA analyses were performed by Illumina sequencing. The V4 variable region of the 16S rRNA genes was amplified by PCR using universal primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011). The DNA library was constructed following the TruSeq DNA library preparation protocol (Illumina, San Diego, CA, USA). Paired-end sequencing (2 × 300 bp) was performed at MR DNA on a MiSeq platform.

2.6. High-throughput sequencing data analysis

Paired-end reads were joined by the MR DNA platform (www.mrdnalab.com, Shallowater, TX, USA). Sequences were analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME v. 1.8.0) (Caporaso et al., 2010). After the sequences were demultiplexed and quality filtered, chimeras were detected and removed using USEARCH 6.1 (Edgar, 2010). The resulting high quality sequences were processed to generate operational taxonomic units (OTUs) with a 97% similarity threshold that were then taxonomically assigned on the Greengenes database (DeSantis et al., 2006; Edgar, 2010).

A variable number of sequences was obtained per sample (range [81,686–143,720]). Therefore, for fair comparison, the sequence number of each sample was randomly normalized to the same sequencing depth (81,000 sequences). The alpha (Chao 1, Shannon's diversity index, phylogenetic diversity (PD)) analyses were performed using QIIME, while beta-diversity analysis (Sorensen dissimilarity index) was performed using QIIME and R 3.0.0 (RCoreTeam, 2013).