

ESCHERICHIA COLI RESISTANCE AND GUT MICROBIOTA PROFILE IN PIGS RAISED WITH DIFFERENT ANTIMICROBIAL ADMINISTRATION IN FEED

**Caroline Pissetti¹, Jalusa Deon Kich², Heather K. Allen³, Claudia Navarrete¹,
Débora da Cruz Payão Pellegrini⁴, Nelson Morés², Marisa Cardoso¹**

¹Departamento de Medicina Veterinária Preventiva, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

²Embrapa Suínos e Aves, Concórdia, SC, Brazil

³Food Safety and Enteric Pathogens Research Unit, National Animal Disease Center, United States Department of Agriculture, Ames, Iowa, USA

⁴Faculdade de Medicina Veterinária, Universidade Federal do Pampa, Uruguai, RS, Brazil

Abstract

Antimicrobials have been widely used in veterinary medicine for disease treatment, disease prevention, and growth promotion. Although, the mechanisms about in-feed additives use are still not completely understood, it is accepted that its use improves feed efficiency by reducing the microbial load in the intestinal tract, thereby reducing the pressure on the immune system and increasing energy availability for the animal. However, disruption to commensal bacterial communities by antibiotics in some cases can increase the gut colonization by pathogenic bacteria. In this sense, the aim of this study was to evaluate the *Escherichia coli* antibiotic resistance and the gut microbiota profile from pigs raised in Brazilian farms with different in-feed antimicrobials protocols. Pigs from four farms with distinct antibiotic usage, including one farm that used no antibiotics, were followed from weaning to finishing, and the frequency of antimicrobial resistance and gut bacterial profile by 16S rRNA gene sequencing were evaluated. The gut microbial community structure was the same among all groups of pigs despite different antibiotic use on the farms; however, the antimicrobial resistance profiles of *E. coli* isolates were different between groups. One farm administered seven antibiotics at different times, and *E. coli* isolates from these pigs showed higher frequency of resistance and multidrug resistance as compared with samples from the farm that did not administer in-feed antimicrobials. The phenotypes included resistance to drugs considered critically important antimicrobial agents in veterinary medicine (ampicillin, ciprofloxacin, florfenicol, sulfonamide and tetracycline) as well as one highly important antibiotic in human medicine (colistin). Resistant *E. coli* strains were screened for the presence of the *mcr-1* gene by PCR. The colistin-resistant strains were positive for the presence of the *mcr-1* gene. These results suggest that although different antibiotic uses on-farm might not impact microbial community structure, it does impact bacterial functions, namely antibiotic resistance. Our results show that prudent use of antimicrobials is important for decreasing selective pressure for antibiotic resistance gene evolution.

Introduction

Antimicrobials have been widely used in veterinary medicine for therapeutic and non-therapeutic purposes. The non-therapeutic use can affect the gut microbiome balance, and may increase the gut colonization by pathogenic and antimicrobial resistant

bacteria. In this sense, meat products can act as vehicles of resistant strains (Chantziaras et al., 2014; Boeckel et al., 2015).

In order to study the effect of continuous in-feed antimicrobial administration on bacterial resistance, many studies used *Escherichia coli* as a biological indicator. It's known that the resistance profile of *E. coli* strains may vary according to in-feed antimicrobial administration protocol (Chantziaras et al., 2014; EFSA, 2015). This administration can also have an effect on the gut microbiota (Looft et al., 2012; Kim et al., 2012; Fleury et al., 2016). However, it is not clear if the gut microbiota structure in pigs from in-feed antimicrobial free farms is different compared to pigs grown in farms, where antimicrobials are used massively. In order to contribute to this knowledge, the aim of this study was to describe the gut microbiota and *E. coli* phenotypic resistance profile in pigs grown in farms adopting different in-feed antimicrobial administration protocols.

Material and methods

Gut microbiota and *E. coli* antimicrobial resistance profile were investigated in four groups of swine submitted to different in-feed antimicrobial administration protocols from 32 to 140 days old. The study was conducted at Embrapa Swine and Poultry (Brazil) and the farms were located at the same region.

Swine groups

- **G1:** Piglets from a very small farrow to finishing operation (462 finished pigs per year), without in-feed antimicrobials administration since 2008.
- **G2:** A subgroup of G1, submitted to three ten-day pulses of in-feed antimicrobial administration: colistin (120 ppm) at the nursery; doxycycline (200 ppm) and tiamulin (120 ppm) at the growing phase; and florfenicol (120 ppm) at the finishing phase.
- **G3:** Piglets from a small farrow to finishing operation (1,100 finished pigs per year). In-feed antimicrobials were used continuously: colistin (133 ppm) from farrow to nursery; halquinol (4000 ppm) at the growing phase; and lincomycin (733 ppm) at the finishing phase.
- **G4:** Piglets from a medium farrow to finishing operation (28,800 finished pigs per year). In-feed antimicrobials were used continuously: florfenicol (60 ppm) and norfloxacin (200 ppm) in nursery (first week), colistin (200 ppm) and neomycin (200 ppm) until end of nursery stage; halquinol (120 ppm) and tiamulin (150 ppm) at growing stage; tylosin (80 ppm) at finishing stage.

Sampling

Six piglets from three different litters were identified and sampled at four time points: nursery (days 32 and 47 of age); growing (day 63 of age); and finishing (ten days before slaughter) stages. Two producing cycles in each farm were sampled, totaling 183 feces samples.

Microbiota analysis

For this analysis, feces samples from four animals in one sampling cycle were used (n = 64). The fecal DNA was extracted by Power Fecal kit (MoBio Laboratories). The V1-V3 region of bacterial 16S rRNA gene was amplified and sequenced in the MiSeq platform (Illumina). Sequence data analysis was performed with *mothur* software package version 1.34.4. Statistical analysis for taxonomic differences was conducted using the STAMP software. T-test with Storey FDR multiple comparison correction was used to detect significant differences in taxonomic distribution between samples, with the unclassified reads retained in the analyses.

Isolation and antimicrobial susceptibility test of *Escherichia coli*

Escherichia coli was isolated on Eosin Methylene Blue Agar (EMB) and one isolate per animal was tested for susceptibility against ten different antimicrobial agents: ampicillin (10 µg); cefotaxime (30 µg); ceftazidime (30 µg); ciprofloxacin (5 µg); chloramphenicol (30 µg); florfenicol (30 µg); gentamicin (10 µg); nalidixic acid (30 µg); sulfonamide (300 µg); and tetracycline (30 µg), according to CLSI (VET01-S2 and M100S documents). Antimicrobial resistance frequencies in the groups were compared by the chi-square test (χ^2) with 95% of confidence. In addition, Marascuilo procedure was performed to test the differences between categories. For colistin, the Minimal Inhibitory Concentration (MIC) was determined. All isolates were also screened for the presence of *mcr-1* gene by PCR (Liu et al., 2016).

Results and discussion

Microbiota analysis produced a total of 853,301 reads with an average of 488 bases (280 to 582). From them, 164,351 reads were used for assessing bacterial diversity. Among the OTUs 99.16% were identifiable, and the majority was assigned to the phyla *Firmicutes* (65.43%) and *Bacteroidetes* (27.98). These groups have been identified as part of the gut microbiota in pigs (Looft et al., 2012; Kim et al., 2015; Mach et al., 2015). At the genus level, *Prevotella* and *Ocillibacter* followed by *Bacteroides* and *Lachnospiracea* were the most frequent groups. *Prevotella*, which belongs to the phylum *Bacteroidetes*, is the most frequent genus in pig gut microbiota (Looft et al., 2012; Mach et al., 2015). This genus has been associated with feed fermentation and provision of energy to the host (Lamandella et al., 2011). *Ocillibacter*, which belongs to the phylum *Firmicutes*, ranged from 17.64 to 19.92% of all classifiable genera identified in the four pig groups. *Ocillibacter* has been reported as a low frequent genus in the gut microbiota of adult pigs. On the contrary, this genus together with *Bacteroides* may predominate in piglets, due to their ability of utilization of oligosaccharides present in the milk (Park et al., 2014; Mach et al., 2015). Overall, no significant difference on the gut microbiota profile was detected among the four pig groups or between growth stages.

As in other studies, *Escherichia*, which belongs to the phylum *Proteobacteria*, was amongst the least common genera of the gut microbiota. Despite this fact, *E. coli* has been adopted as an antimicrobial resistance indicator in animals (EFSA, 2015). Evaluation of antimicrobial susceptibility test was carried out in 183 *E. coli* isolates (Table 1). Overall, 7.65% were susceptible to all antimicrobials tested and 78.14% were considered multidrug-resistant (MDR) (resistant to ≥ 3 antimicrobial classes). Among the pig groups, G4 presented the highest frequency of MDR strains (22.95%), followed

by G3, G2 and G1 (21.3%, 19.7% and 14.2%, respectively). Significant differences between groups were observed in the resistance to ampicillin, ciprofloxacin, chloramphenicol, florfenicol, sulfonamide and tetracycline ($P < 0.05$).

Table 1. Percentage of antimicrobial resistant *Escherichia coli* strains from pigs subjected to different in-feed antimicrobial administration protocols.

Antimicrobial	Group 1	Group 2	Group 3	Group 4	P value
Ampicillin	32.61 ^a	63.83 ^{a,b}	86.96 ^b	77.27 ^{a,b}	0.001
Cefotaxime	8.70	6.38	2.17	2.27	0.389
Ceftazidime	0.00	4.26	0.00	0.00	0.119
Chloramphenicol	34.78 ^a	48.94	67.39	84.09 ^b	< 0.001
Ciprofloxacin	10.87 ^a	23.40	30.43	43.18 ^b	0.006
Florfenicol	28.26 ^a	34.04	52.17	84.09 ^b	< 0.001
Gentamicin	8.70	19.15	21.74	6.82	0.102
Nalidixic acid	60.87	59.57	54.35	75.00	0.217
Sulfonamide	52.17 ^a	68.09	69.57	84.09 ^b	0.014
Tetracycline	67.39 ^a	70.21 ^c	78.26	97.73 ^{b,d}	0.002

Different letters demonstrate differences between groups by Marascuilo procedure.

Regarding colistin, most *E. coli* strains displaying MICs $8 \geq \mu\text{g}\cdot\text{mL}^{-1}$ belonged to G3 and G4 (Table 2). Among the resistant strains, *mcr-1* gene was detected in 77.5% of them. This is a matter of concern, since this gene may be located in plasmids and be transferred among bacteria, decreasing the colistin efficacy in controlling swine diseases. Moreover, the hazard for humans has been pointed out, because this drug is considered the last option for human treatment (Liu et al., 2016).

Table 2. Distribution of *Escherichia coli* strains from pigs subjected to different in-feed antimicrobial administration protocols, according the Minimum Inhibitory Concentrations (MIC) of colistin.

Groups	<i>E. coli</i> (n)	Colistin MIC ($\mu\text{g}/\text{mL}$)			
		2.0	4.0	8.0	16.0
G1	46	22	22	1	1
G2	47	27	18	2	0
G3	46	13	10	19	4
G4	44	14	17	12	1
Total	183	76	67	34	6

Conclusion

These results suggest that although different antibiotic uses on-farm might not impact microbial community structure, it does impact bacterial functions, namely antibiotic resistance. Our results show that prudent use of antimicrobials is important for decreasing selective pressure for antibiotic resistance gene evolution.

References

- Boeckel T., et al. Global trends in antimicrobial use in food. PNAS, 112(18):5649-54, 2015.
- Chantziaras I., et al. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother, 69:827-34, 2014.
- EFSA. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. EFSA Journal 2015, 178p. 2015.
- Fleury M.A., et al. Impact of two different colistin dosing strategies on healthy piglet fecal microbiota. Res Vet Sci, 107:152-60, 2016.
- Kim H.B., et al. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. Proc Natl Acad Sci USA, 109:15485-90, 2012.
- Kim J., et al., Analysis of swine fecal microbiota at various growth stages. Arc Microbiol, 197:753-59, 2015.
- Lamendella R., et al. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol, 11:103, 2011.
- Liu Y-Y., et al. Emergence of plasmid-mediated colistin resistance mechanism *mcr-1* in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis, 16(2):161-8, 2016.
- Looff T., et al. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci USA, 109:1691-96, 2012.
- Mach N., et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ Microbiol Rep, 7(3):554-69, 2015.
- Park S.J., et al. Characterization of the fecal microbiome in different swine groups by high-throughput sequencing. Anaerobe, 28:157-62, 2014.
- You Y., et al. Learning from agriculture: understanding low-dose antimicrobials as drivers of resistome expansion. Front Microbiol, 5(76-86), 2014.