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Field trial evaluating changes in prevalence and patterns of antimicrobial resistance among *Escherichia coli* and *Enterococcus* spp. isolated from growing broilers medicated with enrofloxacin, apramycin and amoxicillin

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Abstract

The present study investigates, under field conditions, the influence of antimicrobial administration on prevalence and patterns of antimicrobial resistance among *Escherichia coli* and *Enterococcus* spp. isolated from growing broilers. For this purpose, a group of 16 000 commercial broiler chickens was treated with enrofloxacin from day 1 to day 3, gentamicin from day 19 to day 21, and ampicillin from day 26 to day 28. A control group of 16 000 broilers was placed in the same controlled environment poultry house. Fecal (from both groups) and feed samples were collected at regular intervals. Few *E. coli* isolates were obtained from either farm environment or poultry feed samples, while enterococci were found to be ubiquitous among these samples. The frequency of resistance against most antimicrobials tested was significantly higher ($P < 0.05$) in *E. coli* isolated from broilers receiving intermittent antimicrobial pressure than that from non-medicated broilers, whereas in enterococci these differences were only observed among structurally related antimicrobial drugs and over a short period of time. By the time the broilers reached market age (33 days), several multi-resistant *E. coli* and enterococci were detected in the feces of the medicated group. Results suggest that antimicrobial resistance in *E. coli* was mainly medication-dependent, whereas among enterococci, changes observed over time were apparently influenced by factors apart from antimicrobial exposure, namely the resistance organisms previously present in farm environment and those present in feedstuffs.

Keywords: antimicrobials; resistance; *Escherichia coli*; enterococci; poultry.

1. Introduction

Historically, the assessment of the biological consequences associated with antimicrobial use in food animals was restricted to zoonotic enteropathogens. During the last decade, there has been an increasing awareness about the effects of antimicrobial drugs on resistance in animal commensal flora, such as *Escherichia coli* and enterococci (van den Bogaard et al., 2002; McDermott et al., 2005; Diarra et al., 2007; Smith et al., 2007). Antimicrobial resistance determinants, generated and/or amplified at the farm level, interfere with therapeutic efficiency (increasing morbidity and mortality associated with disease outbreaks), and may be later transferred to other animals or humans either through direct contact, contamination of meat or, more indirectly, through environmental pathways (Dancer, 2004). Broiler production is unique among animal husbandry, as their fast growing cycle (30 to 42 days) and their high stocking density (14 to 26 birds m^{-2}) result in no tolerance for disease episodes. Thus, preventive mass medication of the growing broilers, administering drugs at therapeutic levels for short periods of time is carried out. Shortly after European Union banned the use of growth promoters in animal production, there were some increases in morbidity and mortality among farm animals that were controlled by a proportional increase in therapeutic and preventive administration of antimicrobials (McEwen and Fedorka-Cray, 2002). This has created serious concerns because the majority of these antimicrobials are used in human medicine, or at least are structurally related.

To better understand the selective pressure exerted by antimicrobials under field conditions, this study examined the effects on development and persistence of antimicrobial resistance among commensal *E. coli* and *Enterococcus* spp. isolated from broiler chickens medicated during their rearing cycle with enrofloxacin, gentamicin and amoxicillin.

2. Materials and Methods

2.1. Animals, housing and treatments

A controlled environment poultry house with 1 600 m^2 area was selected for this study. Inside, 32 000 broiler chicks originating from the same breeder were divided in two groups on a random basis: (i) Medicated group (M) (n = 16 000) medicated with enrofloxacin (Baytril 10%® - Bayer, Carnaxide, Portugal), gentamicin (Gentamicina Oral® - Vetlima, Lisboa, Portugal) and amoxicillin (Paracilina SP® - Intervet, Mem Martins, Portugal); (ii) Control group (C) (n = 16 000). Isolated by a barrier, both groups shared exactly the same rearing, environmental and sanitary conditions. Medicated and control group were fed the same commercial corn-soy meal broiler diet, containing anticoccidial products: narasin at 100 mg kg^{-1} in starter and grower diets and salinomycin at 60 mg kg^{-1} in finisher.

The birds in the medicated group were given, for a period of three days, therapeutic doses of enrofloxacin (23.8 mg kg^{-1} of live weight), gentamicin (26.0 mg kg^{-1}) and amoxicillin (25.1 mg kg^{-1}) in their drinking water when they were 1, 19 and 26 days old, respectively. The antimicrobial concentrations in drinking water for these treatments were as follows: enrofloxacin (50 mg l^{-1}), gentamicin (115 mg l^{-1}) and amoxicillin (140 mg l^{-1}).

Both compartments had independent drinking systems and exterior entrances. Strict biosecurity measures were observed, implying the use of disposable boots and coveralls before each room entry. The broiler house was geographically remote from others and operated under the principle of all-in/all-out; prior to flock placement, the broiler house was thoroughly cleaned and disinfected and was left empty for 14 days

before the flock placement. The previous history of antimicrobial usage was known for this commercial farm.

Once a day, the animals were observed by a veterinarian for checking general health status and for any clinical symptoms of disease, to verify feed and water supply, and to check the proper functioning of heating and ventilation systems.

2.2. Sample collection

Fresh broiler feces were collected randomly along two main diagonals of the broiler compartments, from both medicated and control groups, at study days 4, 9, 14, 11, 22, 25, 29 and 33.

To investigate transmission of *E. coli* and enterococci between consecutive broiler flocks, one day before chicks arrival (day -1), 12 cotton swabs of rearing equipment and inner surfaces of the poultry house were obtained. Additionally, one composite sample of pine shavings (used as bedding) was collected from the transport vehicle and one sample of drinking water (1 000 ml) was obtained from the main water reservoir. Cloacal swabs from 100 broiler chicks were collected at arrival (day zero), before the chickens were placed inside the two shed compartments. Feed samples were also obtained from the storage silo at the time of fecal sampling of broiler groups.

2.3. Isolation and confirmation of enterococci and E.coli

Twenty five grams of each sample (poultry feces and feed) were suspended in 225 ml of Buffered Peptone Water (Oxoid, Basingstoke, United Kingdom) (BPW) and directly plated onto Tergitol BCIG Agar (Biokar Diagnostics, Beauvais, France) (Tergitol) and Kanamycin Aesculin Azide Agar Base (Oxoid) (KAA). To facilitate isolation of *E. coli* and enterococci from feed and poultry house environment samples,

enrichment was performed in BPW (1:10 dilution) and incubated at 37 °C for 16 hours before plating.

Tergitol and KAA agar plates were incubated at 37 ºC for 24 hours and then, after careful examination with a binocular dissecting microscope (magnification, × 20), a maximum of 26 *E. coli* and 12 *Enterococcus* spp. characteristic colonies were selected for susceptibility tests to twelve and ten antimicrobial agents, respectively. *E. coli* confirmation and presumptive identification of the genus *Enterococcus* spp. were performed as previously described (Martins da Costa et al., 2007).

One hundred milliliters of drinking water were, at each time, filtered through a 0.45 µm-pore-size membrane filters (Pall Corporation). The filters were then placed on TBX agar (Biokar Diagnostics) and Slanetz & Bartley Medium (Oxoid) (SB) agar plates and then incubated at 44 °C for 24 hours and 37 °C for 48 hours for detection/enumeration of *E. coli* and *Enterococcus* spp., respectively.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using disk diffusion assay following the guidelines of Clinical and Laboratory Standards Institute (CLSI 2007), using the following antimicrobial drugs (Oxoid) for enterococci: ampicillin (AMP, 10 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (ERY, 30 µg), gentamicin (GEN, 120 µg), quinupristin-dalfopristin (Q-D, 15 µg), nitrofurantoin (NIT, 300 µg), rifampicin (RIF, 5 µg), tetracycline (TET, 30 µg) and vancomycin (VAN, 30 µg); and for *E. coli*: amoxicillin/clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), apramycin (APR, 15 µg), cephalothin (CEF, 30 µg), chloramphenicol (CHL, 30 µg), enrofloxacin (ENR, 5 µg), gentamicin (GEN, 10 µg), kanamycin (KAN,

20 µg), nitrofurantoin (NIT, 300 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg) and trimethoprim/sulfamethoxazole (SXT, 25 µg).

Reference strains *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were included as control. Interpretation of the diameter of the inhibition zone was made according to the recommendations of CLSI and the manufacturer in the case of apramycin (Lilly Farma, Algés, Portugal). Organisms considered intermediate by this method were recorded as sensitive.

2.5. Data analysis

Statistical analysis was carried out using the SPSS version 15.0 for windows. Differences in the prevalence of antimicrobial resistance between both groups were assessed by the Chi-Square test. Alternatively, we used the Fisher's exact test. The null hypothesis that antimicrobial resistance occurred equally in medicated and non-medicated broilers was tested by the Mann-Whitney test. *P* values less than an alpha of 0.05 (Probability of Type I Error) were considered significant throughout this study.

3. Results

No infectious disease outbreak was observed in either group, avoiding non-planned antibiotic treatments.

One feed sample (starter diet) and one environmental sample (swab collected from nipple drinkers) was positive for *E. coli* isolation. All strains were resistant to tetracycline, co-trimoxazol, streptomycin and kanamycin. Only one strain, isolated from the rearing equipment, was resistant to ampicillin and enrofloxacin, whereas no resistance was found against gentamicin.

No cultivable *E. coli* or enterococci were obtained from either drinking water or pine shavings used as bedding, while enterococci were widely distributed in the broiler chicken environment and also in the feed samples. These strains were predominantly tetracycline-resistant. No resistance was found against gentamicin or ciprofloxacin, and only two isolates, obtained from the farm environment, were resistant to ampicillin.

3.1. E. coli

Twenty-six *E. coli* isolates were obtained from cloacal swabs taken from day-old chick and none of these isolates displayed resistance to cephalothin, kanamycin, apramycin, chloramphenicol and enrofloxacin. However, resistance to these antimicrobial drugs were intermittently found in control broilers during the rearing period. Within this group, no resistance against amoxicillin/clavulanic acid was found and resistance rates to ampicillin, tetracycline, trimethoprim/sulfamethoxazole and streptomycin revealed a slight decrease during the rearing period (Table 1).

For the medicated group, the prevalence of resistance was considerably increased upon exposure to the three antimicrobials. Enrofloxacin use selected for *E. coli* with resistance to structurally unrelated antimicrobials, such as the beta-lactams (ampicillin and cephalothin), aminoglycosides (streptomycin and kanamycin), trimethoprim/sulfamethoxazole and chloramphenicol. However, 11 days after the end of enrofloxacin use, only enrofloxacin and tetracycline resistance frequencies were significantly different between both groups. After medication with gentamicin, enrofloxacin resistance frequency had increased again to 100 % and then dropped, progressively, until the end of the rearing period. The more pronounced decrease in the overall resistance five days after antimicrobial treatment was found for ampicillin, as a

result of the emergence of more sensitive strains having resistance phenotypes equivalent to those found in non-medicated broilers.

Four days after the placement of day-old chicks, were found 14 new resistance patterns in *E. coli* isolated from the control group (Table 2). This shift in resistance profiles remained until the end of the study with the gradual isolation of new patterns and, simultaneously, with the "return" of early resistance patterns obtained from cloacal swabs collected from chicks and broiler feces.

Profiles of multi-resistance were more frequent and diverse in the medicated group; among 208 *E. coli* strains isolated in this group, 47, 20, 37, and 9 displayed simultaneous resistance to six (13 patterns), seven (seven patterns), eight (five patterns) and nine (three patterns) antimicrobials, respectively, while in the control group only two *E. coli* strains (two patterns) showed resistance to more than six antimicrobials. The most frequent resistance pattern in control broilers was TET^R (n = 38), whereas in broilers receiving intermittent antimicrobial pressure it was $AMP^RCEF^RTET^RSXT^RSTR^RKAN^RCHL^RENR^R$ (n = 25).

3.2. Enterococci

Comparing resistance rates in enterococci isolated from feces collected from both groups, significant differences were only found for ciprofloxacin, gentamicin, ampicillin and erythromycin ($P < 0.05$) (Table 3). However, after the first medication, and throughout the study, no enterococcal isolates were susceptible to the full range of antimicrobial drugs tested even though sensitive strains were isolated from the cloacal swabs of the day-old chicks. As was the case with *E. coli*, multi-resistant enterococci had only persisted in medicated broilers.

Correspondence between resistance patterns found in growing broilers and those found in cloacal swabs from day-old chicks, feed diets and farm environment prior to birds placement was, respectively, 26.0, 34.4 and 57.3 % for the medicated birds and 44.8, 72.9 and 82.3 % for the control group (Table 4).

None of the seven enterococci which displayed resistance to gentamicin was isolated neither from control group nor from medicated broilers before the use of gentamicin. While among ampicillin-resistant strains, there were three isolated from control group and only five, out of 26 found in medicated broilers, were obtained after the medication with amoxicillin. Additionally, it was found a greater incidence of resistance to ampicillin after gentamicin medication rather than after amoxicillin use. There were no strains exhibiting vancomycin resistance.

3.3. E. coli and enterococci

The overall resistance to all drugs tested reached the highest level after medication with enrofloxacin for *E. coli* and gentamicin for enterococci. One day after enrofloxacin, gentamicin and ampicillin had been used, 100, 58 and 100 % of *E. coli* were resistant to these drugs, respectively, whereas 75, 42 and 42 % of the enterococci were resistant to ciprofloxacin, gentamicin and ampicillin.

After the first medication, and throughout the study, no enterococci and only one *E. coli* revealed susceptibility to all antimicrobial drugs tested, whereas simultaneous resistances against fluoroquinolones, gentamicin and ampicillin were detected in two and 43 isolates of *Enterococcus* spp. and *E. coli*, respectively.

Regarding enterococci, only ampicillin resistance was co-selected through gentamicin medication between days 19 and 21, while in *E. coli* significant differences in resistance incidence between groups had often occurred, without a direct selective

pressure imposed by the use of the same antimicrobial drugs or even by structurally related ones.

At the end of the study, there was a reduction in the differences in resistance profiles in the enterococcal isolates.

4. Discussion

Under field conditions, it is impossible to control the numerous factors that interact in the development and persistence of resistance. Thus, this study was mainly focused in visualizing the selective effects of the sequential medication of a broiler flock with enrofloxacin, gentamicin and amoxicillin on the susceptibility profile of a representative gram-positive (enterococci) and gram-negative enteric bacteria (*E. coli*).

Enterococci were widely distributed in the broiler chicken environment and also in feed samples. The isolation of 11 different phenotypes of multi-resistant enterococci from environmental swabs indicates that there was significant carry-over of resistant enterococci from previous batches of broilers housed in this shed. Previous investigations reported the long-lasting persistence of multi-resistant enterococci in broiler house structure or on production equipment, as they are able to survive several rounds of cleaning and disinfection (Chaslus-Dancla et al., 1987; Heuer et al., 2002).

Though of lesser magnitude, multi-resistant *E. coli* strains were also recovered from the farm environment. The antimicrobial prescription protocol included: enrofloxacin after day-old chicks placement, for prevention of early chick mortality and to reduce possible colonization or horizontal spread of pathogens; gentamicin to stabilize the gut flora promoting a greater uniformity of growth; and, finally, amoxicillin to suppress *Clostridium perfringens* growth and to reduce microbial action on dietary nutrients. In vivo, enrofloxacin is converted to a more potent intermediate

273 product, ciprofloxacin and both agents are completely cross-resistant (van den Bogaard
274 et al., 2001; Yan and Gilbert, 2004). In general, all antimicrobials administered were
275 extremely efficient in selecting for an increase in resistance and their use resulted in the
276 emergence of new resistance phenotypes. This shift in profile was more evident among
277 fecal *E. coli* and it continued beyond the period of antimicrobial exposure. This could
278 be explained by the well known capacity of *E. coli* to exchange resistance genes, as
279 earlier observed in commercial broilers (Bass et al., 1999; Smith et al., 2007), coupled
280 with changes in *E. coli* community structure, i.e. selective enrichment of previously non
281 detected phenotypes.

282 Since antimicrobials were used successively, it was difficult to elect the "more
283 selective" antimicrobial. However, following medication with enrofloxacin, there was a
284 marked change of the resistance patterns found in *E. coli* strains isolated from day-old
285 chicks by enrofloxacin-resistant strains. In the present study, only one enrofloxacin-
286 resistant *E. coli* was detected in the broiler house environment; however two previous
287 studies have described the propensity for some resistant strains to persist in the farm
288 environment and colonize new flocks (Diarra et al., 2007; Diarrassouba et al., 2007). As
289 fluoroquinolones had been used to treat two previous batches of chickens in the shed,
290 this could have contributed to the presence of enrofloxacin-resistant *E. coli* in all fecal
291 samples collected in both groups. However, it was clear from the results that exposure
292 to Gentamicin and amoxicillin were the driving forces in maintaining high frequency of
293 resistance to enrofloxacin during the second half of the rearing period of medicated
294 broilers. The strength of the co-selection effect was particularly evident with the *E coli*
295 isolates and it is probable that it contributed to the emergence and persistence of multi-
296 resistant strains, including resistance to structurally unrelated antibiotics.

297 It was found a greater incidence of resistance to ampicillin after gentamicin
298 medication, than following amoxicillin use. In addition, 37 of the 42 different
299 phenotypes which displayed resistance to ampicillin were isolated from medicated
300 broilers before the use of ampicillin. The majority of the resistance profiles included
301 either resistance to enrofloxacin or gentamicin to resistance to both antimicrobial drugs.
302 The extent of these co-selective effects is consistent with phenotypic or genotypic
303 evidence found in other studies (Berge et al., 2005; Harada et al., 2007).

304 Antimicrobial resistance in control birds not exposed to antimicrobials also
305 exhibited interesting changes over time, as early reported by others (Diarra et al., 2007;
306 Pleydell et al., 2007). Although *E. coli* strains sensitive to all antimicrobial, as well as
307 tetracycline-resistant ones, persisted only in these non-medicated broilers, multi-
308 resistant patterns were also found in the absence of selective antimicrobial pressure.

309 Similarly, as shown by the results obtained within the control group regarding to
310 enterococci, antimicrobials were not necessarily the cause of the increase in resistance
311 against ampicillin, tetracycline, erythromycin and nitrofurantoin. This shift in resistance
312 frequencies might be explained by colonization of the broilers' intestines with strains
313 found in the feed and in the broiler house environment, since their resistance patterns
314 were often equivalent. These data are in agreement with previously published studies,
315 which indicated that farm indigenous microbiota and feed-associated bacteria may have
316 a greater influence on the prevalence of antimicrobial resistance than antimicrobial
317 usage (McDermott et al., 2005; Smith et al., 2007).

318 While enterococci found in the feed were not expected to be as well adapted to
319 the broiler intestine as those present within the farm environment, their massive
320 numbers could compensate for this, considering the great amount of feed ingested
321 (almost 3.340 kg^{-1} bird) during the broilers rearing period. In an earlier study, involving

22 broiler feed samples, it was observed that cultivable enterococci had ranged between 1.32 and 3.99 log CFU g^{-1} (Martins da Costa et al., 2007).

Among enterococci, the overall resistance to all drugs tested reached the highest level after medication with gentamicin. This was unexpected, as enterococci are intrinsically resistant to aminoglycosides at a low level (8-128 µg ml^{-1}) (Klare et al., 2003). However, gentamicin is a relatively stable drug (Yan and Gilbert, 2004). Thus, considering that gentamicin given orally is poorly absorbed from the gastrointestinal tract and that intestine content represents less than one tenth of the live broiler weight, fecal gentamicin concentrations high enough to favor the selective enrichment of gentamicin-resistant enterococci may have occurred. Furthermore, salinomycin and narasin or any other stress factor within the intestinal environment, could damage the enterococcal cell wall, increasing gentamicin penetration into enterococci and thus contributing to a significant elimination of susceptible strains.

In contrast to the emergence of gentamicin-resistant enterococci, the appearance of ampicillin-resistant strains had no chronological relationship with the use of amoxicillin. This was most likely due to co-selection by the antimicrobials used earlier, as 43 % and 29 % of these patterns displayed resistance against ciprofloxacin and gentamicin, respectively.

Cessation of antimicrobial use did not appear to result in an immediate reduction in antimicrobial resistance. The short growing cycle of modern broilers might not provide sufficient time for resistance determinants to be lost once antimicrobial treatment ceases.Furthermore, we must be aware of the potential risk of spreading these multi-resistant strains to poultry farmers or even beyond farm boundaries, exposing humans via food chain and/or environmental pathways.

346 The understanding of the association between antimicrobial use in food
347 producing animals and the development and persistence of resistant commensal bacteria
348 may help us to manage critical decisions concerning use of antimicrobials in these
349 animals.
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Table 1: Evolution and comparison of the percentage of antibiotic resistance in 26 isolates of *Escherichia coli* obtained from feces samples collected from medicated (M) and non-medicated (C) broilers on days 4, 9, 14, 18, 22, 25, 29 and 33. Medicated birds were treated with Enrofloxacin from day 1 to day 3, Gentamicin from day 19 to day 21, and Ampicillin from day 26 to day 28.

Antibiotics[a]	Days																							
	4			9			14			18			22			25			29			33		
	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*
AMP	58	96	0.001	35	92	<0.001	46	65	0.163	46	58	0.405	46	61	0.266	38	85	0.001	38	100	<0.001	31	81	<0.001
AMC	0	4	1.000	0	0	-	0	0	-	0	0	-	0	11	0.235	0	0	-	0	0	-	0	0	-
CEF	4	61	<0.001	0	42	<0.001	11	35	0.048	11	8	1.000	19	23	0.734	15	27	0.308	11	65	<0.001	11	42	0.012
TET	96	100	1.000	69	96	0.024	77	81	0.734	54	85	0.016	69	96	0.024	69	100	0.004	73	100	0.010	65	81	0.211
SXT	38	85	0.001	46	81	0.010	31	61	0.026	31	46	0.254	27	77	<0.001	35	58	0.095	46	61	0.266	27	42	0.244
STR	46	88	0.001	42	88	<0.001	42	50	0.578	35	46	0.397	35	58	0.095	23	69	0.001	15	92	<0.001	15	35	0.109
KAN	11	50	0.003	19	54	0.010	15	27	0.308	0	15	0.110	8	8	1.000	0	23	0.023	8	73	<0.001	11	19	0.703
GEN	4	11	0.610	0	11	0.235	4	8	1.000	0	0	-	0	58	<0.001	0	50	<0.001	4	35	0.005	0	23	0.023
APR	0	0	-	0	8	0.490	0	4	1.000	0	0	-	0	58	<0.001	0	15	0.110	4	15	0.350	4	11	0.610
CHL	15	73	<0.001	8	27	0.140	4	27	0.050	8	27	0.140	0	15	0.110	0	19	0.051	4	31	0.024	0	27	0.010
NIT	0	11	0.235	0	8	0.490	0	11	0.235	0	8	0.490	0	0	-	0	19	0.051	4	19	0.191	0	4	1.000
ENR	50	100	<0.001	19	100	<0.001	15	88	<0.001	11	65	<0.001	8	100	<0.001	19	81	<0.001	15	65	<0.001	8	54	<0.001

[a]Abbreviations: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cephalothin; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; APR, apramycin; CHL, chloramphenicol; NIT, nitrofurantoin; ENR, enrofloxacin.

Table 2: Number of antimicrobial resistance patterns in *Escherichia coli* isolates from day-old chick meconium and broiler feces of medicated and non-medicated control broilers.

Antimicrobial[a] resistance pattern	Day-old chicks	Control group								Medicated group							
		Day								Day							
		4	9	14	18	22	25	29	33	4	9	14	18	22	25	29	33
AMP TET SXT NIT	7																
AMP TET STR GEN	6							1									
AMP TET SXT STR	5			1													
none	4	1	6	3	7	4	4	3	4				1				
TET	3	6	1	4	1	4	9	3	10			1	1				1
AMP TET SXT	1			2			2	1	2								
TET SXT STR		3	5		3	1	1										
AMP TET SXT STR ENR		3	1		1	1	4		1	2	4	1	2		1		
AMP TET STR KAN		2	4	4		2		1	2				1		1	6	3
TET ENR		1	1									1	3				
AMP TET		1	2	2		1									1		
AMP TET ENR		1		1	1			1			1		2		2	1	1
AMP TET STR ENR		1									1	1					
AMP TET STR CHL ENR		1			1							2	4		1		
AMP TET STR KAN ENR		1															
AMP TET SXT ENR		1	1					1		1		3					
AMP TET SXT CHL		1	2														
AMP TET SXT GEN CHL ENR		1								2		1		2	2		3
AMP TET SXT STR CHL ENR		1								2	1		2				
AMP CEF TET ENR		1											1				3
TET SXT			2	2	3	4	1	7					2				1
TET SXT STR KAN			1														
TET SXT STR ENR				3		1	1										
AMP CEF				2		4	2	1	1								5
AMP TET SXT STR CHL				1	1								1				
AMP CEF TET STR				1	3	1											
AMP					5		2	1									
AMP TET STR						3											
AMP CEF TET								2	1								
SXT								1	3			2	2				
TET STR KAN								1									
TET SXT APR CHL ENR								1						1			1
AMP SXT STR NIT ENR								1									
TET SXT APR ENR									1			1		6			1
AMP STR KAN									1								
AMP CEF TET SXT STR KAN CHL ENR										9	6	2				7	1
AMP CEF TET SXT STR KAN NIT ENR										2	1		1		1	3	
TET STR ENR										1				1			

TET STR CHL ENR			1							
AMP TET SXT STR KAN CHL ENR			1		1					
AMP CEF TET STR CHL ENR			1		1					1
AMP CEF TET STR GEN ENR			1	1			4	2		
AMP CEF TET SXT STR CHL ENR			1							
AMP CEF TET SXT STR KAN CHL NIT ENR			1							
AMP AMC CEF TET SXT STR CHL ENR			1							
AMP TET SXT STR KAN ENR				5	2	1				
AMP TET SXT STR GEN APR ENR				2			5	2		
AMP CEF TET SXT STR KAN ENR				2	2					
TET NIT ENR				2	2	1				1
TET STR NIT ENR					1			3		
AMP CEF TET SXT ENR					1					
AMP CEF TET SXT STR KAN GEN NIT ENR					1			1	2	
TET SXT ENR							1			
TET SXT GEN APR CHL ENR							1			
AMP TET STR GEN APR ENR							1			
AMP CEF TET SXT STR GEN ENR							1		1	
AMP AMC SXT STR ENR							1			
AMP AMC TET SXT STR KAN ENR							1			
AMP AMC CEF TET SXT STR KAN APR ENR							1			
AMP TET SXT STR GEN APR								2		1
TET STR GEN								1		
AMP TET SXT KAN ENR								1		
AMP TET SXT STR GEN ENR								1		1
AMP TET SXT STR KAN GEN CHL ENR								1		1
AMP CEF TET SXT GEN CHL ENR								1	1	
AMP CEF TET SXT KAN ENR								1		
AMP CEF TET SXT STR ENR								1		
AMP CEF TET STR GEN APR									2	
AMP TET STR GEN APR									1	
AMP TET SXT STR KAN GEN ENR									1	
AMP CEF TET SXT STR GEN APR ENR									1	
AMP CEF TET SXT STR										1

[a] For antimicrobial abbreviations, see Table 1, footnote a.

Table 3: Evolution and comparison of the percentage of antibiotic resistance in 12 isolates of *Enterococcus* spp. obtained from feces samples collected from medicated (M) and non-medicated (C) broilers on days 4, 9, 14, 18, 22, 25, 29 and 33. Medicated birds were treated with Enrofloxacin from day 1 to day 3, Gentamicin from day 19 to day 21, and Ampicillin from day 26 to day 28.

Antimicrobials[a]	Days																							
	4			9			14			18			22			25			29			33		
	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*	C	M	*P*
AMP	0	0	-	17	0	0.478	8	17	1.000	0	17	0.478	0	58	0.005	0	25	0.217	0	42	0.037	0	17	0.478
VAN	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-	0	0	-
Q-D	0	25	0.217	8	8	1.000	0	0	-	17	8	1.000	0	0	-	0	17	0.478	0	0	-	17	0	0.478
TET	75	67	1.000	75	92	0.590	75	75	1.000	100	92	1.000	100	75	0.217	92	92	1.000	92	100	1.000	83	100	0.478
RIF	42	42	1.000	0	25	0.217	25	8	0.590	8	25	0.590	0	8	1.000	8	25	0.590	17	8	1.000	0	0	-
ERY	25	58	0.098	50	67	0.408	58	92	0.155	42	75	0.098	50	100	0.014	58	92	0.155	50	50	1.000	67	75	1.000
GEN	0	0	-	0	0	-	0	0	-	0	0	-	0	42	0.037	0	8	1.000	0	0	-	0	8	1.000
CHL	0	8	1.000	8	0	1.000	0	0	-	0	0	-	0	0	-	0	0	-	0	8	1.000	0	8	1.000
NIT	17	50	0.193	25	42	0.667	42	42	1.000	42	50	0.682	50	75	0.400	50	58	0.682	50	42	0.682	50	50	1.000
CIP	8	75	0.001	8	67	0.009	0	33	0.093	8	50	0.069	8	25	0.590	8	33	0.317	8	25	0.590	0	0	-

[a]Abbreviations: AMP, ampicillin; VAN, vancomycin; Q-D, quinupristin-dalfopristin; TET, tetracycline; RIF, rifampicin; ERY, erythromycin; GEN, gentamicin; CHL, chloramphenicol; NIT, nitrofurantoin; CIP, ciprofloxacin.

Table 4: Number of antimicrobial resistance patterns in *Enterococcus* spp. isolated from the house environment (Farm, n=24), feed samples (n=61), day-old chicks meconium (n=12) and feces of non-medicated control broilers (n=96) and medicated broilers (n=96). Medicated birds were treated with Enrofloxacin from day 1 to day 3, Gentamicin from day 19 to day 21, and Ampicillin from day 26 to day 28.

Resistance patterns	Farm	Feed	Day-old chicks	Control group								Medicated group								No. total
				day								day								
				4	9	14	18	22	25	29	33	4	9	14	18	22	25	29	33	
TET NIT	3	33					3	4	3	1	3				1				1	52
TET	7	8	2	4	4		3	2		2			2	1	1			4	1	43
TET ERY	4	2	1	1	1	2	3	4	4		2		2	4	1		1	1	5	39
TET ERY NIT	2	5		1		4		1	2	5	3				1		2		2	28
none		4	5		2	2			1	1	1									21
AMP TET ERY NIT	2				2	1								2		4	3	3	1	18
ERY NIT CIP	1			1	1							4	1	3		2	1			14
TET RIF	1	1	1	3		2			1	2		1								13
TET ERY NIT CIP								1	1			2	3		1					8
TET RIF ERY		3	2											1						8
TET ERY CIP										1				1	3		1	1		7
Q-D TET ERY	1		1		1						2									6
TET RIF NIT		5													1					6
TET RIF CIP												2	2					1		5
RIF				2		1														3
ERY	2										1									3
Q-D TET RIF ERY							1										2			3
TET NIT CIP							1										1			2
TET ERY GEN																2				2
Q-D TET RIF												2								2
AMP TET ERY CHL NIT																		1	1	2
AMP TET ERY GEN NIT																2				2
TET ERY CHL					1															1
Q-D TET	1																			1
Q-D TET ERY NIT							1													1
RIF ERY																1				1
RIF ERY NIT CIP															1					1
TET GEN NIT																			1	1
TET RIF ERY NIT CIP													1							1
TET RIF ERY GEN CIP																	1			1
Q-D TET ERY CIP													1							1
Q-D TET ERY CHL CIP												1								1
AMP TET NIT CIP																		1		1
AMP TET ERY GEN NIT CIP																1				1
AMP TET RIF ERY CIP															1					1
AMP Q-D TET ERY NIT CIP															1					1

[a] For antimicrobial abbreviations, see Table 3, footnote a.