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The effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal *E. coli* in broilers

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Abstract

The effect of a competitive exclusion product (Aviguard®) on the selection of fluoroquinolone resistance in poultry was assessed in vivo in the absence or presence of fluoroquinolone treatment.

Two experiments using a controlled seeder-sentinel animal model (2seeders:4sentinels per group) with one-day-old chicks were used. For both experiments, as soon as the chicks were hatched, the animals of two groups were administered Aviguard® and two groups were left untreated. Three days later, all groups were inoculated with an enrofloxacin-susceptible commensal \(E.\ coli\) strain. Five days after hatching, two animals per group were inoculated either with a bacteriologically-fit or a bacteriologically non-fit enrofloxacin-resistant commensal \(E.\ coli\) strain. In experiment 2, all groups were orally treated for three consecutive days (Day 8-10) with enrofloxacin. Throughout the experiments, faecal excretion of all inoculated \(E.\ coli\) strains was determined on days 2-5-8-11-18-23 by selective plating (via spiral plater). Linear mixed models were used to assess the effect of Aviguard® on the selection of fluoroquinolone resistance.

The use of Aviguard® (p<0.01) reduced the excretion of enrofloxacin-resistant \(E.\ coli\) when no enrofloxacin treatment was administered. However, this beneficial effect disappeared (p=0.37) when the animals were treated with enrofloxacin. Similarly, bacterial fitness of the enrofloxacin-resistant \(E.\ coli\) strain used for inoculation had an effect (p<0.01) on the selection of enrofloxacin resistance when no treatment was administered, whereas this effect was no longer present when enrofloxacin was administered (p =0.70).
Thus, enrofloxacin treatment cancelled the beneficial effects from administrating Aviguard® in one-day-old broiler chicks and resulted in an enrofloxacin-resistant flora.

**Research Highlights**

- Use of Aviguard® was assessed *in vivo* on the selection of enrofloxacin resistance.
- Without enrofloxacin, Aviguard® reduced the selection of enrofloxacin resistance.
- When enrofloxacin was administered, it cancelled the beneficial effect of Aviguard®.

**Introduction**

Antimicrobial agents have been used globally for more than six decades in animal production. Yet, bacterial populations have responded by evolving resistance mechanisms against all used agents (Levin, 2001). This has led to a ban of antimicrobial agents used as growth promoters in the EU (European Regulation No. 1831/2003) and worldwide calls for more prudent use of antimicrobials (van den Bogaard *et al.*, 2002; Dibner & Richards, 2005). Especially in poultry meat production, high levels of antimicrobial resistance are found due to extensive antimicrobial use (Castanon, 2007). Fluoroquinolones have been widely used in veterinary medicine and especially in broiler production for more than two decades (Gouvea *et al.*, 2015). Despite their efficacy, the use of fluoroquinolones in veterinary medicine is controversial (Landoni & Albarellos, 2015) and increased fluoroquinolone resistance rates in both human and animal bacterial isolates have led to restrictions in its use (Rushton *et al.*, 2014; AFSCA/FAVV, 2016) or complete withdrawal from
the market (FDA, 2005). Fluoroquinolone treatment can affect intestinal microbiota and select for fluoroquinolone resistant strains in both commensal and pathogenic bacteria (Pepin et al., 2005). Fluoroquinolone resistance can be associated with a biological fitness cost via the acquisition of mutations (Melnyk et al., 2015) that can negatively affect the bacterial metabolism (Lindgren et al., 2005; Gualco et al., 2007; Park et al., 2013). However cost-free mutations (Luo et al., 2005) or compensatory mutations that ameliorate fitness cost have also been described (Marcusson et al., 2009; Andersson & Hughes, 2010).

Several strategies have been proposed to reduce the prevalence of antimicrobial resistance including optimising antimicrobial use (Paterson et al., 2016) or using alternatives to antibiotics (Joerger, 2003; Allen et al., 2013). There has been an increasing interest in using non-antibiotic feed additives, including competitive exclusion (CE) products (Mountzouris et al., 2009; Ducatelle et al., 2015). In this study, Aviguard® (Microbial Developments Limited, Malvern, UK), a commercial CE product, was tested using a standardized animal model for its potential effect in preventing the excretion and spread of fluoroquinolone resistance. Aviguard® comprises of a partially-defined freeze-dried mixture of live commensal bacteria that were derived from the gut microbiota of specific-pathogen-free adult chickens (Abudabos, 2013). In principle, CE products are administered to newly hatched chicks in order to quickly induce the formation of a diverse yet stable intestinal microbiota and subsequently to prevent pathogens colonizing the gut (Nurmi & Rantala, 1973). The majority of studies has focused on the role of CE in preventing the introduction of pathogenic strains such as Salmonella spp. (Rantala & Nurmi, 1973; Nurmi et al., 1992; Vandeplas et al., 2010), Campylobacter spp. (Stern et al., 2001), E. coli (Hofacre et al., 2002) and Clostridium perfringens (Dahiya et al., 2006;
Abudabos, 2013). However, little research has been performed to evaluate the effect of CE products to prevent the introduction (Hofacre, et al., 2002; Nuotio et al., 2013) and the spread (Ceccarelli et al., 2017) of antimicrobial resistance.

The current research therefore aimed at quantifying the effect of a commercially available CE product on the selection and spread of fluoroquinolone resistance in commensal *E. coli* in broilers, using a well-defined and controlled experimental *in vivo* model and taking into account the effect of enrofloxacin treatment.

**Materials and methods**

**Ethics**

*In vivo* experiments were compliant with all relevant institutional and European standards for animal care and experimentation. All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2015/118, EC2016/61).

**Table 1. Strains used in this paper**

<table>
<thead>
<tr>
<th>Strain used</th>
<th>Parental</th>
<th>Bacteriological fitness</th>
<th>Resistance to enrofloxacin</th>
<th>Resistance to rifampicin (marker)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strain</td>
<td>(compared to its parental strain)</td>
<td>Strain MIC (mg/L)</td>
<td>Strain MIC (mg/L)</td>
</tr>
<tr>
<td>E. coli IA31</td>
<td>E. coli IA2</td>
<td>Fit</td>
<td>Susceptible 0.032</td>
<td>Resistant &gt; 256</td>
</tr>
<tr>
<td>E. coli IA50</td>
<td>E. coli IA31</td>
<td>Non-fit</td>
<td>Resistant 32</td>
<td>Resistant &gt; 256</td>
</tr>
<tr>
<td>E. coli IA66</td>
<td>E. coli IA31</td>
<td>Fit</td>
<td>Resistant 32</td>
<td>Resistant &gt; 256</td>
</tr>
</tbody>
</table>

**Bacterial strains**

*E. coli* strain IA31, a previously characterized (Chantziaras et al., 2017) non-pathogenic spontaneous rifampicin-resistant and enrofloxacin-susceptible strain, was used as the reference strain for this study (Table 1). No detection of plasmid mediated quinolone resistance (PMQR) genes was observed using a PCR protocol.
described by Robicsek et al. to detect for any qnrA, qnrB or qnrS determinants (Robicsek et al., 2006), and by Park et al. to detect for aac(6')-lb-cr determinant (Park et al., 2013). Starting from IA31, a bacteriologically non-fit spontaneous enrofloxacin-resistant strain (E. coli IA50) and a bacteriologically-fit spontaneous enrofloxacin-resistant strain (E. coli IA66) were derived as described before (Chantziaras et al., 2017). In short, bacterial fitness was assessed with in vitro growth competition assays between each resistant strain and the parental susceptible strain. The bacteriologically non-fit strain (IA50) was outcompeted by the parental strain (IA31) and as a result its population decreased over time compared to the population of the parental strain. On the other hand, bacterial populations of the bacteriologically fit strain (IA66) and of the parental strain (IA31) were similar throughout the duration of the in vitro competition assays.

Prior to each experiment, the content of the CE product (Aviguard®, Lallemand Animal Nutrition UK, Worcestershire) was resuscitated and plated on McConkey agar no.3 (Oxoid Ltd, Basingstoke, UK). After overnight aerobic incubation, lactose-positive isolates were purified and subsequently identified by standard biochemical testing. Susceptibility testing was performed on all E. coli isolates using the gradient strip method according to the guidelines of the manufacturer (E-test®, BioMérieux, Marcy l’Etoile, France). Although a new foil laminate sachet was used in each experiment, both sachets belonged to the same batch (No 1440).

**Eggs, chickens, housing and welfare**

Embryonated 17-day-old eggs were collected under aseptic conditions from a commercial poultry hatchery (Vervaek-Belavi, Belgium). The eggs were disinfected with the use of a formaldehyde gas mixture at the hatchery, but after transportation to the experimental facilities, they were additionally dipped in 5% H₂O₂ for 10 seconds.
After drying for 20-25 seconds they were further incubated in two separate sanitized hatching cabinets. Each cabinet was allocated in a separate, previously decontaminated HEPA-filtered stable that was used for the actual experiment as well. After hatch, the chicks were housed in groups of four (control groups) or six (tested groups) animals in 1m² disinfected plastic boxes in these HEPA-filtered stables. All necessary biosecurity measures were taken to avoid the introduction of irrelevant strains and cross-contamination between groups as described before (Chantziaras, et al., 2017). Six groups were used in each experiment (groups A, B, D and E consisted of six animals and groups C and F consisted of four animals). In total, 64 chickens were used in this study (32 per experiment). The birds received daily 16 hours of light, and had free access to autoclaved food and bottled water. Each bird was individually numbered to allow for individual fecal collection. All birds were observed on a daily basis and any clinical sign of disease was registered. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium).

**Experimental setup**

The experimental set up was identical for both experiments (Fig 1). As soon as the chicks were hatched, all animals from Groups A and D were orally treated with Aviguard®. Aviguard® was suspended in water according to the manufacturer’s instructions and 0.2 ml was administered per chick with a needle-less sterile syringe. On Day 3, all animals (Groups A to F) were orally inoculated with the enrofloxacin-susceptible *E. coli* strain IA31. The inoculum contained approximately $10^8$ *E. coli* colony forming units (cfu)/ml and each animal received 0.2 ml of this inoculum via needle-less sterile syringe. On day 5, two animals per group (from Groups A, B, D and E) were inoculated with an enrofloxacin-resistant *E. coli* strain. The
bacteriologically-fit enrofloxacin-resistant *E. coli* strain (IA66) was inoculated in the seeders of group A and B. The bacteriologically non-fit enrofloxacin-resistant *E. coli* strain IA50 was inoculated in the seeders from group D and E. For both strains, the inoculum contained approximately $10^8$ *E. coli* cfu/ml and each animal received 0.2 ml of this inoculum via needle-less sterile syringe. After inoculation, these animals (seeders) were re-introduced in their respective pens with the four remaining animals of each group (sentinel animals).

In experiment 2, groups A, B, D, E additionally received 10 mg/kg bodyweight enrofloxacin via drinking water (Baytril™ 10% oral solution, Bayer AG, Leverkusen, Germany) for 3 days (day 8 to day 10 after hatching).

The sampling procedure was identical for both experiments. In total, six fresh faecal samplings took place in each experiment. After the first sampling at day 2 after hatching, there was a second sampling shortly before the inoculation of the seeder animals on day 5 after hatching. A third sampling occurred on day 8 after hatching (for experiment 2, this was shortly before the start of the enrofloxacin treatment). The remaining sampling days took place on days 11, 18 and 23 after hatching. Each sample was collected individually as previously described (Chantziaras, et al., 2017).

**Bacteriological enumeration in faecal samples**

The faecal content was serially ten-fold diluted in phosphate buffered saline solution ($10^{-1}$ to $10^{-4}$). The spiral plating technique was used to enumerate the different *E. coli* populations (Eddy Jet, IUL Instruments, Barcelona, Spain).

All serial dilutions were plated on i) unsupplemented McConkey agar plates, ii) rifampicin-supplemented (100 mg/L) McConkey (rMC) agar plates and iii) enrofloxacin-supplemented (0.25 mg/L) and rifampicin-supplemented (100 mg/L) McConkey (erMC) agar plates. Preliminary testing showed that coliforms obtained
from Aviguard® were not able to grow either on the rMC or the erMC agar plates. Since the enrofloxacin-susceptible strain cannot grow on the enrofloxacin-supplemented plates, these plates were used to differentiate between the inoculated strains and allowed for the calculation of the ratio of susceptible and resistant strains.

After inoculation, all plates were placed in an aerobic incubator set at 37°C ± 1°C and examined after 24h ± 3h for the presence of colonies. The colonies were counted on plates ideally having 20 - 200 colonies per plate and the number of cfu/g of faeces was calculated.

**Statistical analysis**

**Excretion of enrofloxacin-resistant E. coli strains**

Statistical analysis was performed separately for each experiment. The dependent variable used was the proportion of the enrofloxacin-resistant colonies in the total number of retrieved E.coli. The proportion data were transformed using the arcsine square root transformation so as to follow bivariate normal distributions more accurately. All animals from Groups A, B, D, E were included in the model. The fixed factors that were studied were the following: bacteriological fitness (fit, non-fit), Aviguard® treatment (Aviguard®, No Aviguard®), seeders (seeders, sentinels). Linear mixed models were used (IBM SPSS Statistics for Windows, version 24.0, Armonk, NY). Each animal was listed as subject, and sampling as repeat. An autoregressive covariance matrix of the first order was used for the repeated covariance structure.

To simultaneously assess all the aforementioned effects, results from all sampling days (except Day 2, Day 5) were included. All potential fixed factors were first tested univariately. Only variables with a P-value < 0.2 were selected to be included in the multivariate model. The model was build according to the stepwise forward
procedure. All potential two-way interactions between significant fixed factors were tested. Bonferroni correction was used to adjust confidence intervals for multiple comparisons. The significance level was set at $P \leq 0.05$.

**Results and Discussion**

**Evaluation of the experimental setup**

All *E. coli* isolates obtained from plating Aviguard® on McConkey agar were shown to be susceptible for both enrofloxacin and rifampicin and they were not able to grow on both enrofloxacin-supplemented and enrofloxacin/rifampicin-supplemented McConkey agar plates. None of the animals showed any signs of disease throughout the duration of both experiments. The *E. coli* strains that were used, successfully colonized the gastrointestinal tract of the animals as shown in Figs 2 – 4. The use of these isogenic strains allowed for direct comparisons between the *in vivo* experiments since differences between the fit and non-fit strains can be attributed to the point mutations leading to resistance. The enrofloxacin susceptible *E. coli* IA31 strain showed wild-type MIC levels for enrofloxacin and the presence of PMQR genes was ruled out as discussed in a previous study (Chantziaras et al., 2017). Also, when studying the data from the control groups from both experiments (Fig 4), only colonies from the enrofloxacin-susceptible inoculated strain (IA31) were isolated from all sampling days. This indicates that no cross-contamination between the groups in the different pens occurred.

Concerning the *E. coli* populations during the *in vivo* experiments, a relative decline was observed over time (Figs 2 – 4) but a sufficient number of *E. coli* colonies were retrieved in all samples until the end of the experiment, thus allowing for a meaningful statistical analysis of the obtained data. This decline is expected as several bacteria
from phyla such as Firmicutes, Bacteroides are Proteobacteria are expected to persist in the gastrointestinal tract of chickens and thus compete and reduce the inoculated *E. coli* population (Pan & Yu, 2014). At day 2 after hatching, *E. coli* was only detected in the groups A and D, receiving Aviguard® at hatch, in both experiments. This illustrates that the protocol successfully prevented the appearance of *E. coli* isolates—at least in detectable levels— in non-Aviguard®-treated groups.

Moreover, preparatory in vivo experiments (data not shown) that were performed to measure the autochthonous flora of *E. coli* and test the biosecurity level of the stables resulted in non-detectable levels of *E. coli* in the feces of chickens throughout the duration of the experiments (day 1 until day 14). Therefore it can be concluded that the *E. coli* isolates obtained in the Aviguard®-treated groups were actually originating from the competitive exclusion product.

*Aviguard® reduces excretion and transmission of fluoroquinolone-resistant* *E. coli*

The use of Aviguard® resulted in a lower faecal excretion of enrofloxacin-resistant *E. coli* bacteria (*p*<0.01) compared to the groups that did not receive Aviguard®.

Additionally, and in agreement with previous studies (Hughes, 2014; Redgrave *et al.*, 2014; Melnyk, *et al.*, 2015), fitness had a significant effect (*p*<0.01) on the transmission of enrofloxacin resistance in the absence of enrofloxacin treatment (Table 2). More specifically, the animals of the groups that were inoculated with the non-fit enrofloxacin-resistant (IA50) strain showed a lower faecal excretion of enrofloxacin resistant *E. coli* (*p*<0.01) compared to the groups that were inoculated with the fit enrofloxacin-resistant strain. Even though seeders seemed to excrete more enrofloxacin-resistant *E. coli* than sentinel animals, this difference was statistically not significant (*p*=0.137).
The current findings indicate that the use of Aviguard® reduces the excretion of both the fit and non-fit strains. Nonetheless, further repetitions of the experiment and a higher group size might have enabled us to calculate the transmission ratio of the enrofloxacin-resistant strains and thus precisely measure the spread of these strains as well.

In the absence of treatment (Fig 2), the *E. coli* population originating from Aviguard® was predominant and largely prevented the establishment and spread of both the bacteriological-fit or the bacteriological non-fit enrofloxacin-resistant. This is in agreement with the results of a recent study focusing on the effect of the use of Aviguard® on the epidemiology of extended-spectrum cephalosporin (ESC)-resistant *E. coli* (Ceccarelli et al., 2017). Ceccarelli et al. (2017) showed that the excretion and transmission of an ESC-resistant strain in the absence of antimicrobial treatment was reduced in chickens pretreated with Aviguard®. These results suggest that indeed the use of Aviguard® may have a beneficial effect on the spread of resistant strains. However, while in the present work and in the work of Ceccarelli et al. (2017) the introduction of the resistant strains took place a few days after the administration of Aviguard®, this is not always the case in the field.

It has been shown that day-old chickens can “inherit” bacterial isolates from their parents (Bortolaia *et al.*, 2010; Mezhoud *et al.*, 2016) and the role of parent breeding stocks in disseminating antimicrobial-resistant bacteria to their progeny has been highlighted in various studies focusing mainly on β-lactam-resistance (Persoons *et al.*, 2011; Börjesson *et al.*, 2013; Mo *et al.*, 2014; Mo *et al.*, 2016; Projahn *et al.*, 2016), quinolone resistance (Petersen *et al.*, 2006; Börjesson *et al.*, 2016) or both (Bortolaia, *et al.*, 2010). Thus, in order to successfully intervene and reduce the transmission and excretion of resistant strains, the use of CE products (e.g.
Aviguard®) should probably take place in earlier instances than administrating in one-
day-old chicks. This is confirmed by the report that administration of a CE product
after the inoculation of an ESC-resistant E. coli strain did not result in a reduction of
the transmission of the resistant strain (Ceccarelli, et al., 2017). As a consequence it
is believed that the use of Aviguard® in great-grandparent and parent stocks, the in
ovo inoculation of Aviguard® or spraying of Aviguard® on embryonated eggs, before
exposure to antimicrobial treatments or resistant strains, are promising as these
applications could potentially help more to reduce the prevalence of antimicrobial
resistant determinants. Yet, further studies should be performed to test the latter
under field conditions.

Fluoroquinolone treatment abolishes Aviguard® effects on excretion and transmission
of fluoroquinolone resistant E. coli strains

After the administration of enrofloxacin, both enrofloxacin-resistant strains managed
to spread to all sentinel animals and became highly prevalent in faecal samples until
the end of the experiment (Fig 3). No significant effect of Aviguard use (p=0.366) or
bacterial fitness (p=0.704) in the spread of fluoroquinolone resistance was observed
(Table 2). Moreover, seeder and sentinel animals showed no significantly different
faecal excretion of enrofloxacin-resistant E. coli strains (p=0.870). This suggests that
the inoculated enrofloxacin-resistant E. coli strains outcompeted both the susceptible
strain (IA31) and the E. coli population that originated from the CE product (Groups
A, D) under the selective pressure provided by enrofloxacin treatment.

Comparing the results from both experiments in this study (Fig 5), a clear difference
is seen among the groups that received enrofloxacin treatment and those that did
not. The effect of treatment had by far the biggest impact on the excretion of
fluoroquinolone resistance effectively overriding all other effects. To overcome this
Table 2. Linear mixed models performed per experiment to assess the effects of bacterial fitness, Aviguard® and EF-resistant strain transmission (Seeders versus Sentinels).

<table>
<thead>
<tr>
<th>Categorical variable / Parameter</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>Std. Error</td>
</tr>
<tr>
<td><strong>Aviguard®</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-0.075</td>
<td>0.028</td>
</tr>
<tr>
<td>No (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial fitness</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-fit</td>
<td>-0.073</td>
<td>0.028</td>
</tr>
<tr>
<td>Fit (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EF-resistant strain transmission (Seeders)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seeders</td>
<td>0.046</td>
<td>0.054</td>
</tr>
<tr>
<td>Sentinels (ref.)</td>
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<tr>
<td><strong>Aviguard®</strong></td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.094</td>
<td>0.102</td>
</tr>
<tr>
<td>No (ref.)</td>
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<td></td>
</tr>
<tr>
<td><strong>Bacterial fitness</strong></td>
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<td></td>
</tr>
<tr>
<td>Non-fit</td>
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<td>0.104</td>
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<tr>
<td>Fit (ref.)</td>
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<tr>
<td><strong>EF-resistant strain transmission (Seeders)</strong></td>
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<td></td>
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<tr>
<td>Seeders</td>
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<td>0.111</td>
</tr>
<tr>
<td>Sentinels (ref.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The dependent variable used was the (arcsine square root transformed) proportion of the enrofloxacin-resistant colonies to the sum of the resistant and the susceptible colonies.

effect, it has been proposed that Aviguard® could be used after the antimicrobial treatment period to re-establish a susceptible gut microbiota (Stavric & Komegay, 2008). However, it is questionable if the later inoculated commensal bacterial microbiota could successfully replace the highly prevalent resistant microbiota that is expected to be found after antimicrobial treatment as recent findings indicate otherwise (Ceccarelli, et al., 2017).
Conclusions

In the absence of treatment, a commercially-available competitive exclusion product (Aviguard®) reduced the faecal excretion and transmission of enrofloxacin resistant *E. coli* strains in chicks. When enrofloxacin was administered to the animals, enrofloxacin-resistant strains quickly disseminated within the groups effectively overriding all other effects. Thus, to keep the beneficial effect of this competitive exclusion product, antimicrobial treatment should be avoided as much as possible.

Disclosure statement

None to declare.
References


Figure 1. Schematic plan (a. and b.) of the experimental setup for both experiments. On Day 1, all animals from Groups A and D were orally administered a competitive exclusion product (Aviguard®). On Day 3, all animals from all groups were orally inoculated with a bacteriologically-fit enrofloxacin-sensitive E. coli strain (IA31). On Day 5, two animals (seeders) from Group A and two from Group B received orally a bacteriologically-fit enrofloxacin-resistant E. coli strain (IA66). Similarly, on Day 5, two animals from Group D and two from Group E received orally a non-fit enrofloxacin-resistant E. coli strain (IA50). Transmission of enrofloxacin-resistant strains from seeders (shown in red) to the other animals from each group (sentinels) was studied. Each stable contained a control group (inoculated with E. coli IA31 only). In experiment 2, groups A, B, D and E received enrofloxacin oral (via drinking water) treatment (Baytril™ 10% oral solution). Treatment period lasted 3 days (day 7 to day 9). The treatment started right after the second sampling took place. The treatment dose (10 mg/kg bodyweight) was calculated based on the recommended therapeutic protocol of the company (Bayer AG, Leverkusen, Germany) and the drinking water medication was prepared daily.  

Figure 2. Experiment 1 results. The y-axis presents the (log-scaled) E. coli cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). Results on x-axis are
presented separately for seeders and sentinels although this distinction is meaningful only after day 5. ‘total’ depicts
the total *E. coli* population, ‘inoculated strains’ refers to the population of both inoculated strains (Groups A & B: Strains IA31 & IA66 and Groups D & E: Strains IA31 & IA50) and ‘enro res’ indicates the population of enrofloxacin-resistant *E. coli*.\(^1\): enrofloxacin-resistant

Figure 3. Experiment 2 results. The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). Results on x-axis is presented separately for seeders and sentinels although this distinction is meaningful only after day 5. ‘total’ depicts the total *E. coli* population, ‘inoculated strains’ refers to the population of both inoculated strains (Groups A & B: Strains IA31 & IA66 and Groups D & E: Strains IA31 & IA50) and ‘enro res’ informs of the population of enrofloxacin-resistant *E. coli*. Enrofloxacin treatment was administered orally to all animals for three consecutive days from Day 8 to Day 10 (blue dotted line).\(^1\): enrofloxacin-resistant, \(^2\): all animals were treated with enrofloxacin.

Figure 4. Control groups results for experiments 1 and 2. On Day 3, all animals from all groups were orally administered a rifampicin-resistant *E. coli* strain (IA31). The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). ‘Total’ depicts the total *E. coli* population, ‘inoculated’ refers to the population of IA31 isolate. ‘enro res’ informs of the population of enrofloxacin-resistant *E. coli*.

Figure 5. Prevalence of enrofloxacin-resistant *E. coli* strains. On Day 1, all animals from Groups A were orally inoculated with Aviguard\(^\text{®}\). On Day 3, all animals from all groups were orally administered a rifampicin-resistant *E. coli* strain (IA31). On Day 5, a bacteriologically-fit strain (IA66) was introduced in groups A and B and a bacteriologically non-fit strain (IA50) was introduced in groups D and E right after the end of the sampling process. Additionally in experiment 2, enrofloxacin treatment was administered orally to all animals for three consecutive days from Day 8 (after sampling process) to Day 10 (blue dotted text box). The y-axis presents the percentage of enrofloxacin-resistant *E. coli* to the total *E. coli* population (retrieved from individual droppings) per group per sampling day. \(^1\): enrofloxacin, \(^2\): enrofloxacin-resistant.