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 a 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

Strain used	*Parental strain*	*Bacteriological fitness (compared to its parental strain)*	*Resistance to enrofloxacin*		*Resistance to rifampicin (marker)*	
			Strain	MIC (mg/L)	Strain	MIC (mg/L)
***E. coli* IA31**	*E. coli* IA2	Fit	Susceptible	0.032	Resistant	> 256
***E. coli* IA50**	*E. coli* IA31	Non-fit	Resistant	32	Resistant	>256
***E. coli* IA66**	*E. coli IA31*	Fit	Resistant	32	Resistant	>256

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')-Ib-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 (Vervaeke-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% H_2O_2 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 $1m^2$ 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

241 from phyla such as Firmicutes, Bacteroides are Proteobacteria are expected to
242 persist in the gastrointestinal tract of chickens and thus compete and reduce the
243 inoculated *E.coli* population (Pan & Yu, 2014). At day 2 after hatching, *E. coli* was
244 only detected in the groups A and D, receiving Aviguard® at hatch, in both
245 experiments. This illustrates that the protocol successfully prevented the appearance
246 of *E. coli* isolates –at least in detectable levels- in non-Aviguard®- treated groups.
247 Moreover, preparatory *in vivo* experiments (data not shown) that were performed to
248 measure the autochthonous flora of *E. coli* and test the biosecurity level of the
249 stables resulted in non-detectable levels of *E. coli* in the feces of chickens throughout
250 the duration of the experiments (day 1 until day 14). Therefore it can be concluded
251 that the *E. coli* isolates obtained in the Aviguard®-treated groups were actually
252 originating from the competitive exclusion product.

253 *Aviguard® reduces excretion and transmission of fluoroquinolone-resistant* E. coli
254 The use of Aviguard® resulted in a lower faecal excretion of enrofloxacin-resistant *E.*
255 *coli* bacteria ($p<0.01$) compared to the groups that did not receive Aviguard®.
256 Additionally, and in agreement with previous studies (Hughes, 2014; Redgrave *et al.*,
257 2014; Melnyk, et al., 2015), fitness had a significant effect ($p<0.01$) on the
258 transmission of enrofloxacin resistance in the absence of enrofloxacin treatment
259 (Table 2). More specifically, the animals of the groups that were inoculated with the
260 non-fit enrofloxacin-resistant (IA50) strain showed a lower faecal excretion of
261 enrofloxacin resistant *E. coli* ($p<0.01$) compared to the groups that were inoculated
262 with the fit enrofloxacin-resistant strain. Even though seeders seemed to excrete
263 more enrofloxacin-resistant *E. coli* than sentinel animals, this difference was
264 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; Borjesson *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.

290 Aviguard®) should probably take place in earlier instances than administrating in one-
291 day-old chicks. This is confirmed by the report that administration of a CE product
292 after the inoculation of an ESC-resistant *E. coli* strain did not result in a reduction of
293 the transmission of the resistant strain (Ceccarelli, et al., 2017). As a consequence it
294 is believed that the use of Aviguard® in great-grandparent and parent stocks, the *in*
295 *ovo* inoculation of Aviguard® or spraying of Aviguard® on embryonated eggs, before
296 exposure to antimicrobial treatments or resistant strains, are promising as these
297 applications could potentially help more to reduce the prevalence of antimicrobial
298 resistant determinants. Yet, further studies should be performed to test the latter
299 under field conditions.

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

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

311 Comparing the results from both experiments in this study (Fig 5), a clear difference
312 is seen among the groups that received enrofloxacin treatment and those that did
313 not. The effect of treatment had by far the biggest impact on the excretion of
314 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).

		Univariate analysis			*Multivariate analysis (final model)*		
	Categorical variable / Parameter	*Estimate*	*Std. Error*	*P-value*	*Estimate*	*Std. Error*	*P-value*
Experiment 1	**Aviguard®**						
	Yes	-0.075	0.028	0.015	-0.074	0.024	0.007
	No (ref.)						
	Bacterial fitness						
	Non-fit	-0.073	0.028	0.018	-0.072	0.025	0.008
	Fit (ref.)						
	EF-resistant strain transmission (Seeders)						
	Seeders	0.046	0.054	0.157			
	Sentinels (ref.)						
Experiment 2	**Aviguard®**						
	Yes	0.094	0.102	0.366			
	No (ref.)						
	Bacterial fitness						
	Non-fit	-0.040	0.104	0.704			
	Fit (ref.)						
	EF-resistant strain transmission (Seeders)						
	Seeders	-0.019	0.111	0.863			
	Sentinels (ref.)						

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

Abudabos, A.M. (2013). Use of a competitive exclusion product (Aviguard (R)) to prevent clostridium perfringens colonization in broiler chicken under induced challenge. *Pakistan Journal Zoology,* 45, 371-376.

AFSCA/FAVV. (2016). Royal decree of 21/07/2016 relative to the terms of use of drugs by veterinarians and animal management in Belgium. Available in https://www.famhp.be/sites/default/files/downloads/AR-KB-2006-12-14_en.pdf in Dutch, French and German (date last accessed: 01 February 2018).

Allen, H.K., Levine, U.Y., Looft, T., Bandrick, M. & Casey, T.A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in microbiology,* 21, 114-119.

Andersson, D.I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology,* 8, 260-271. doi: 10.1038/nrmicro2319

Borjesson, S., Bengtsson, B., Jernberg, C. & Englund, S. (2013). Spread of extended-spectrum beta-lactamase producing Escherichia coli isolates in Swedish broilers mediated by an incl plasmid carrying bla(CTX-M-1). *Acta Veterinaria Scandinavica,* 55, 3. doi: 10.1186/1751-0147-55-3

Börjesson, S., Guillard, T., Landén, A., Bengtsson, B. & Nilsson, O. (2016). Introduction of quinolone resistant Escherichia coli to Swedish broiler population by imported breeding animals. *Veterinary microbiology,* 194, 74-78.

Bortolaia, V., Bisgaard, M. & Bojesen, A.M. (2010). Distribution and possible transmission of ampicillin-and nalidixic acid-resistant Escherichia coli within the broiler industry. *Veterinary microbiology,* 142, 379-386.

Castanon, J.I.R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science,* 86, 2466-2471. doi: 10.3382/ps.2007-00249

Ceccarelli, D., van Essen-Zandbergen, A., Smid, B., Veldman, K.T., Boender, G.J., Fischer, E.A., et al. (2017). Competitive exclusion reduces transmission and excretion of extended-spectrum

beta-lactamase producing Escherichia coli in broilers. *Applied and environ microbiol*ogy, 83, e03439-16 doi: 10.1128/AEM.03439-16

Chantziaras, I., Boyen, F., Callens, B. & Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of antimicrobial chemotherapy,* 69, 827-834. doi: 10.1093/jac/dkt443

Chantziaras, I., Smet, A., Haesebrouck, F., Boyen, F. & Dewulf, J. (2017). Studying the effect of administration route and treatment dose on the selection of enrofloxacin resistance in commensal Escherichia coli in broilers. *Journal of antimicrobial chemotherapy*, 72, 1991-2001. doi: 10.1093/jac/dkx104

Dahiya, J., Wilkie, D., Van Kessel, A. & Drew, M. (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology,* 129, 60-88.

Dibner, J.J. & Richards, J.D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science,* 84, 634-643.

Ducatelle, R., Eeckhaut, V., Haesebrouck, F. & Van Immerseel, F. (2015). A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. *Animal,* 9, 43-48. doi: 10.1017/S1751731114002584

FDA (2005). Withdrawal of Enrofloxacin for Poultry. Available in http://www.fda.gov/AnimalVeterinary/SafetyHealth/RecallsWithdrawals/ucm042004.htm (date last time accessed: 01 February 2018)

Gouvea, R., Dos, S.F.F., Aquino, M.H.C.D. & Pereira, V.L.D. (2015). Fluoroquinolones in Industrial Poultry Production, Bacterial Resistance and Food Residues: a Review. *Brazilian Journal of Poultry Science,* 17, 1-10. doi: 10.1590/1516-635x17011-10

Gualco, L., Schito, A.M., Schito, G.C. & Marchese, A. (2007). In vitro activity of prulifloxacin against Escherichia coli isolated from urinary tract infections and the biological cost of prulifloxacin

391 resistance. *International Journal of Antimicrobial Agents,* 29, 679-687. doi:
392 10.1016/j.ijantimicag.2007.01.009
393 Hofacre, C.L., Johnson, A.C., Kelly, B.J. & Froyman, R. (2002). Effect of a commercial competitive
394 exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic
395 Escherichia coli in day-old broiler chickens. *Avian Diseases,* 46, 198-202. doi: Doi
396 10.1637/0005-2086(2002)046[0198:Eoacce]2.0.Co;2
397 Hughes, D. (2014). Selection and evolution of resistance to antimicrobial drugs. *IUBMB life,* 66, 521-
398 529.
399 Joerger, R. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and
400 bacteriophages. *Poultry science,* 82, 640-647.
401 Landoni, M.F. & Albarellos, G. (2015). The use of antimicrobial agents in broiler chickens. *Veterinary*
402 *Journal,* 205, 21-27. doi: 10.1016/j.tvjl.2015.04.016
403 Levin, B.R. (2001). Minimizing potential resistance: A population dynamics view. *Clinical Infectious*
404 *Diseases,* 33, S161-S169. doi: Doi 10.1086/321843
405 Lindgren, P.K., Marcusson, L.L., Sandvang, D., Frimodt-Moller, N. & Hughes, D. (2005). Biological cost
406 of single and multiple norfloxacin resistance mutations in Escherichia coli implicated in
407 urinary tract infections. *Antimicrobial Agents and Chemotherapy,* 49, 2343-2351. doi:
408 10.1128/Aac.49.6.2343-2351.25005
409 Luo, N.D., Pereira, S., Sahin, O., Lin, J., Huang, S.X., Michel, L., et al. (2005). Enhanced in vivo fitness of
410 fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection
411 pressure. *Proceedings of the National Academy of Sciences of the United States of America,*
412 102, 541-546. doi: DOI 10.1073/pnas.0408966102
413 Marcusson, L.L., Frimodt-Moller, N. & Hughes, D. (2009). Interplay in the Selection of
414 Fluoroquinolone Resistance and Bacterial Fitness. *Plos Pathogens,* 5. doi: ARTN
415 e100054110.1371/journal.ppat.1000541

Melnyk, A.H., Wong, A. & Kassen, R. (2015). The fitness costs of antibiotic resistance mutations. *Evolutionary applications,* 8, 273-283.

Mezhoud, H., Chantziaras, I., Iguer-Ouada, M., Moula, N., Garmyn, A., Martel, A., et al. (2016). Presence of antimicrobial resistance in coliform bacteria from hatching broiler eggs with emphasis on ESBL/AmpC-producing bacteria. *Avian Pathology*, 45, 1-30.

Mo, S.S., Kristoffersen, A.B., Sunde, M., Nødtvedt, A. & Norström, M. (2016). Risk factors for occurrence of cephalosporin-resistant Escherichia coli in Norwegian broiler flocks. *Preventive Veterinary Medicine,* 130, 112-118.

Mo, S.S., Norström, M., Slettemeås, J.S., Løvland, A., Urdahl, A.M. & Sunde, M. (2014). Emergence of AmpC-producing Escherichia coli in the broiler production chain in a country with a low antimicrobial usage profile. *Veterinary microbiology,* 171, 315-320.

Mountzouris, K., Balaskas, C., Xanthakos, I., Tzivinikou, A. & Fegeros, K. (2009). Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with Salmonella enteritidis. *British poultry science,* 50, 467-478.

Nuotio, L., Schneitz, C. & Nilsson, O. (2013). Effect of competitive exclusion in reducing the occurrence of Escherichia coli producing extended-spectrum β-lactamases in the ceca of broiler chicks. *Poultry science,* 92, 250-254.

Nurmi, E., Nuotio, L. & Schneitz, C. (1992). The competitive exclusion concept: development and future. *International journal of food microbiology,* 15, 237-240.

Nurmi, E. & Rantala, M. (1973). New aspects of Salmonella infection in broiler production. *Nature,* 241, 210-211.

Pan, D. & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes,* 5, 108-119. doi: 10.4161/gmic.26945

Park, M., Sutherland, J.B., Kim, J.N. & Rafii, F. (2013). Effect of fluoroquinolone resistance selection on the fitness of three strains of Clostridium perfringens. *Microbial Drug Resistance,* 19, 421-427.

442 Paterson, I.K., Hoyle, A., Ochoa, G., Baker-Austin, C. & Taylor, N.G. (2016). Optimising Antibiotic
443 Usage to Treat Bacterial Infections. *Scientific reports,* 6, 37853. doi: 10.1038/srep37853
444 Pepin, J., Saheb, N., Coulombe, M.A., Alary, M.E., Corriveau, M.P., Authier, S., et al. (2005).
445 Emergence of fluoroquinolones as the predominant risk factor for Clostridium difficile-
446 associated diarrhea: a cohort study during an epidemic in Quebec. *Clinical infectious*
447 *diseases,* 41, 1254-1260. doi: 10.1086/496986
448 Persoons, D., Haesebrouck, F., Smet, A., Herman, L., Heyndrickx, M., Martel, A., et al. (2011). Risk
449 factors for ceftiofur resistance in Escherichia coli from Belgian broilers. *Epidemiology and*
450 *Infection,* 139, 765-771. doi: 10.1017/S0950268810001524
451 Petersen, A., Christensen, J.P., Kuhnert, P., Bisgaard, M. & Olsen, J.E. (2006). Vertical transmission of
452 a fluoroquinolone-resistant Escherichia coli within an integrated broiler operation. *Veterinary*
453 *microbiology,* 116, 120-128.
454 Projahn, M., Daehre, K., Roesler, U. & Friese, A. (2016). ESBL-/pAmpC-producing enterobacteria in
455 the broiler hatchery–a mode of pseudo-vertical transmission? *Applied and Environmental*
456 *Microbiology*, AEM-02364.
457 Rantala, M. & Nurmi, E. (1973). Prevention of the growth of Salmonella infantis in chicks by the flora
458 of the alimentary tract of chickens. *British poultry science,* 14, 627-630. doi:
459 10.1080/00071667308416073
460 Redgrave, L.S., Sutton, S.B., Webber, M.A. & Piddock, L.J. (2014). Fluoroquinolone resistance:
461 mechanisms, impact on bacteria, and role in evolutionary success. *Trends in microbiology,*
462 22, 438-445.
463 Robicsek, A., Strahilevitz, J., Sahm, D.F., Jacoby, G.A. & Hooper, D.C. (2006). qnr Prevalence in
464 ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrobial*
465 *Agents and Chemotherapy,* 50, 2872-2874. doi: 10.1128/Aac.01647-05

Rushton, J., J. Pinto Ferreira and K. Stärk (2014), Antimicrobial Resistance: The Use of Antimicrobials in the Livestock Sector. *OECD Food, Agriculture and Fisheries Papers*, 68. doi: 10.1787/5jxvl3dwk3f0-en.

Stavric, S. & Komegay, E.T. (2008). Microbial probiotics for pigs and poultry. In R.J. Wallace, A. Chesson (Eds.). *Biotechnology in Animal Feeds and Animal Feeding*, 205-231. Wiley VCH.

Stern, N.J., Cox, N.A., Bailey, J.S., Berrang, M.E. & Musgrove, M.T. (2001). Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce Salmonella and Campylobacter spp. colonization in broiler chickens. *Poultry Science,* 80, 156-160.

van den Bogaard, A.E., Willems, B., London, N., Top, J. & Stobberingh, E.E. (2002). Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy,* 49, 497-505. doi: DOI 10.1093/jac/49.3.497

Vandeplas, S., Dubois Dauphin, R., Beckers, Y., Thonart, P. & Thewis, A. (2010). Salmonella in chicken: current and developing strategies to reduce contamination at farm level. *Journal of Food Protection,* 73, 774-785.

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. [1]: competitive exclusion, [2]: enrofloxacin

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

494 presented separately for seeders and sentinels although this distinction is meaningful only after day 5. 'total' depicts
495 the total *E. coli* population, 'inoculated strains' refers to the population of both inoculated strains (Groups A & B:
496 Strains IA31 & IA66 and Groups D & E: Strains IA31 & IA50) and 'enro res' indicates the population of
497 enrofloxacin-resistant *E. coli*. [1]: enrofloxacin-resistant
498
499 Figure 3. Experiment 2 results. The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual
500 droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). Results on x-axis is presented
501 separately for seeders and sentinels although this distinction is meaningful only after day 5. 'total' depicts the total *E.*
502 *coli* population, 'inoculated strains' refers to the population of both inoculated strains (Groups A & B: Strains IA31 &
503 IA66 and Groups D & E: Strains IA31 & IA50) and 'enro res' informs of the population of enrofloxacin-resistant *E.*
504 *coli*. Enrofloxacin treatment was administered orally to all animals for three consecutive days from Day 8 to Day 10
505 (blue dotted line).[1]: enrofloxacin-resistant, [2]:all animals were treated with enrofloxacin
506
507 Figure 4. Control groups results for experiments 1 and 2. On Day 3, all animals from all groups were orally
508 administered a rifampicin-resistant *E. coli* strain (IA31). The y-axis presents the (log-scaled) *E. coli* cfu/g faeces
509 (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis).
510 'Total' depicts the total *E. coli* population, 'inoculated' refers to the population of IA31 isolate.'enro res' informs of
511 the population of enrofloxacin-resistant *E. coli*.
512
513 Figure 5. Prevalence of enrofloxacin-resistant *E. coli* strains. On Day 1, all animals from Groups A were orally
514 inoculated with Aviguard®. On Day 3, all animals from all groups were orally administered a rifampicin-resistant *E.*
515 *coli* strain (IA31). On Day 5, a bacteriologically-fit strain (IA66) was introduced in groups A and B and a
516 bacteriologically non-fit strain (IA50) was introduced in groups D and E right after the end of the sampling process.
517 Additionally in experiment 2, enrofloxacin treatment was administered orally to all animals for three consecutive days
518 from Day 8 (after sampling process) to Day 10 (blue dotted text box). The y-axis presents the percentage of
519 enrofloxacin-resistant *E. coli* to the total *E. coli* population (retrieved from individual droppings) per group per
520 sampling day. [1]: enrofloxacin, [2]:enrofloxacin-resistant
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