Epidemiology of antimicrobial resistance in commensal *E. coli*

Focus on selection and spread of fluoroquinolone resistance in broilers

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All men naturally desire knowledge.
(Aristotle, Metaphysics I (980a 21)).

This book is dedicated in the loving memory of my friend Athanasios Karagiannis (1982-2008)
Ghent, Belgium, 3 July 2017

Πάντες ἄνθρωποι τοῦ εἰδέναι ὀρέγονται φύσει. (Αριστοτέλης)
Ευχετήριος πάπυρος από την Ακαδημία Αιανής, το έτος 2000.

Το παρών βιβλίο είναι αφιερωμένο στην μνήμη του αγαπημένου μου φίλου Αθανάσιου Καραγιάννη (1982-2008)
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List Of Abbreviations

aac(6')-Ib-cr: Aminoglycoside 6'-N-acetyl transferase type Ib-cr

ABC transporter : ATP-binding cassette transporter

AM : antimicrobial

AR : antimicrobial resistance

ATP : Adenosine triphosphate

AUC : area under curve

BK : benzalkonium chloride

blaZ gene : beta-lactamase gene

CE concept : competitive exclusion concept

CLSI : Clinical and Laboratory Standards Institute

chlorexidine : CHX

Cmax : drug's peak serum concentration

CMY : cephalomycinase (encoded by bla_{CMY} gene)

czrC : cadmium and zinc resistance protein C

DDDA : defined daily dose animal

DCDA : defined cure dose animal

DNA : Deoxyribonucleic acid

EF : enrofloxacin

EFSA : European Food Safety Authority

Escherichia coli : E. coli

EHEC: enterohemorrhagic *E. coli*

EMA : European Medicines Agency

erm gene : erythromycin resistance methylase gene

ESBL : extended-spectrum beta-lactamases

ESC-resistant *E. coli :* extended-spectrum cephalosporin resistant *E. coli*

ESVAC : European Surveillance of Veterinary Antimicrobial Consumption

EPEC: enteropathogenic *E. coli*

ETEC : enterotoxigenic *E. coli*

EUCAST : European Committee on Antimicrobial Susceptibility Testing

g : gram

gyr gene : gyrase gene

HGT : horizontal gene transfer

int gene : integrase gene

i.m. : intramuscular

IS : insertion sequence

i.v. : intravenous

kg : kilogram

l : liter

LB broth : lysogeny broth (or Luria Bertani medium)

LPS : lipopolysaccharide

mar operon : multiple antibiotic resistance operon

mg : milligram

MIC : minimal inhibition concentration

MDR : multi-drug resistant

mdtABC : multi-drug transporter ABC

ml : milliliter

MLS_B resistance : resistance to macrolides, lincosamides and streptogramin B

MRSA : methicillin-resistant *Staphylococcus aureus*

MPC : mutant prevention concentration

MSW : mutant selection window

pcoA protein : copper resistance gene A

PCR : polymerase chain reaction

PCU : population correction unit

per os : oral

PK/PD : pharmacokinetics/pharmacodynamics

PMQR : plasmid mediated quinolone resistance

QAC : quaternary ammonium compounds

QRDR : quinolone resistance determining region

SCC*mec* : staphylococcal chromosome *mec*

smr gene : staphylococcal multidrug resistance gene

SPI1 : *Salmonella* pathogenicity island -1

SSuT : streptomycin-sulfadiazine-tetracycline

tcrB gene *:* transferable copper-resistance gene B

tetA protein : tetracycline resistance protein A

tetB protein : tetracycline resistance protein B

TLN : triclosan

TMP : Trimethoprim

Tn : transposon

UDDA : used daily dose animal

WHO : World Health Organisation

vanA gene*:* vancomycin resistance gene A

VTEC : verocytotoxigenic *E. coli*

INTRODUCTION

1.1. Effect Of Antimicrobial Use On Antimicrobial Resistance Selection, Spread And Persistence In Farm Animals

Effect Of Antimicrobial Use On Antimicrobial Resistance Selection, Spread And Persistence In Farm Animals

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Abstract

The association between antimicrobial use and antimicrobial resistance (AR), albeit a non-linear one, has been pointed out by numerous studies. A critical lower level of drug consumption is required to trigger the increase of resistance although a further increase beyond a critical upper level of drug consumption does not necessarily result in further increase in AR prevalence. The overall use of antimicrobials clearly plays a role in the selection, spread and persistence of AR. The effect of dose seems to be of particular relevance for mutational AR but, concerning horizontal transfer of resistance, no comparable studies are available to support specific linkages between dosage regimens (*e.g.* low dose versus high dose) and the selection of horizontally-transferred resistance. Also, the route of administration may have some influence, but the effect of different administration routes depends on the antibiotic used, among other things because this is linked to a specific excretion route. Interpretation of the link between antimicrobial use and AR is further complicated by the genetic linkages of different resistance mechanisms. In the fight against AR, avoiding unnecessary antimicrobial use should be priority. Whenever antimicrobials are required, treatment strategy should be carefully planned. The choice of a specific antimicrobial should not be based exclusively on the elimination of the target pathogen, but should equally take into account all aspects aiming at a minimal selection of resistance determinants in the host's microbiota.

Introduction

Antimicrobials are natural, semi-synthetic or synthetic chemical compounds that, through various mechanisms of action, can inhibit bacterial growth or kill bacteria. They are extremely important for treatment of bacterial infections, both in humans and animals. However, efficacy of these drugs is seriously threatened by the emerging of antimicrobial resistance (AR).

Several conditions may favor spread of AR in a bacterial population, including the presence of resistance genes in this population, the fitness of resistant clones, the mobility of resistance genes and the presence of a selection pressure (Schwarz and Chaslus-Dancla, 2001). Fitness of the resistant clone may affect vertical transmission of resistance, while transferability of resistance genes may favor horizontal spread.

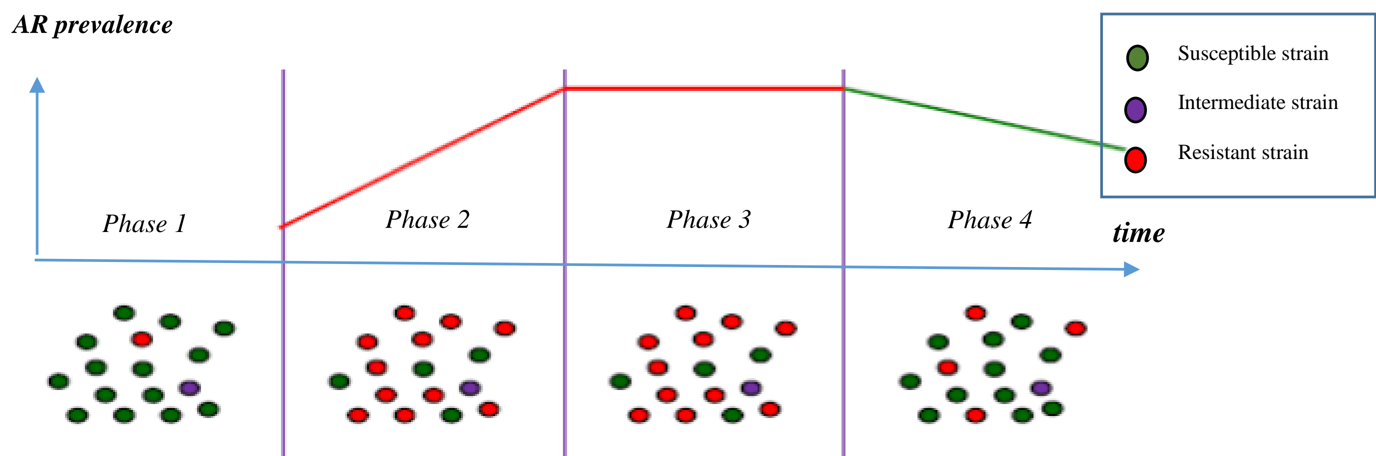

Figure 1. Epidemiology of antimicrobial resistance (AR). Four phases are described: Phase 1. Emergence of AR, Phase 2. Selection and spread of AR, Phase 3. Persistence of AR, Phase 4. Reduction of AR. During Phase 1 the majority of the strains are susceptible. Under selection pressure (e.g. use of antimicrobials) the susceptible strains decrease and the resistant strains become the majority (Phase 2) and persist (Phase 3). In the absence of selection pressure, a reduction of AR prevalence may occur (Phase 4).

The epidemiology of AR is very complex and can be presented as a succession of four phases (Fig. 1). The first phase is the emergence of resistance. It can be assumed that

various mechanisms of antimicrobial resistance already existed in nature, long before the clinical use of these substances, since antimicrobial resistance often serves as a self-defense mechanism of bacteria either against antimicrobial-producing organisms in their surroundings or against antimicrobials produced by these bacteria themselves (Finley et al., 2013; Martins et al., 2013). Yet the anthropogenic use of antimicrobials may contribute to the emergence of AR. Antimicrobials can act as a stress factor for bacteria and may increase the frequency of mutations and recombinations. They may also facilitate resistance gene transfer (Levy and Marshall, 2004; van Hoek et al., 2011). Anthropogenic use of antimicrobials is even more important in the selection and spread phase of AR (phase 2). It will inhibit or even eliminate susceptible bacteria, while less susceptible or resistant organisms will become the predominant population. This is referred to as antimicrobial selection pressure. Once AR is selected for, it may persist in a bacterial population (phase 3). Persistence may need a continuous selection pressure, even though in some cases resistance persists even in the absence of any antimicrobial selection pressure (Andersson and Hughes, 2011). Co- and cross-resistance selection also plays an important role in the persistence of AR. Finally, often in the absence of a selection pressure, reduction (phase 4) of AR may occur, due to the loss or silencing of resistance genes or due to replacement of resistant bacteria by susceptible ones (Andersson and Hughes, 2010).

While numerous articles have reviewed AR mechanisms in various bacterial species, this review will primarily focus on the key aspects of selection, spread and persistence of AR (phases 2 and 3) through antimicrobial use in farm animals (Table 1).

Selection and co-selection exerted by antimicrobial use

Selection pressure exerted by antimicrobial use alters bacterial populations by both selecting for resistant strains and affecting the rate of spread within and between the

exposed animals (Olofsson and Cars, 2007). The most straightforward and best studied link between antimicrobial use and AR is the effect of the use of a specific antimicrobial on the resistance development against that particular agent itself. This has not only been described in bacterial populations *in vitro* (Cloeckaert and Chaslus-Dancla, 2001; Stamey, 1976), but also *in vivo*. At herd-level, several studies showed that bacterial strains often have a decreased susceptibility towards antimicrobial classes administered in the herd (Dorado-Garcia et al., 2016; Dewulf et al., 2007; Persoons et al., 2010). Co-selection also participates in the selection and spread of AR through several mechanisms (Table 2). It renders the relationship between usage and resistance more difficult to interpret (Harada et al., 2008). The two main mechanisms involved in co-selection are cross-resistance and co-resistance. Cross-resistance refers to the selection of resistance to antimicrobial agents by any other antimicrobial of the same antimicrobial class or across different classes of antibiotics with identical mechanisms of action (for instance macrolides, lincosamides and streptogramin B antibiotics). It may refer to the whole class of antibiotics or only to some members, depending on the resistance mechanism. Co-resistance is defined as resistance selection to an antimicrobial through the usage of unrelated antimicrobials, for instance as a result of linkage of multiple resistance genes on the same genetic element. The latter will result in the collective positive selection of all genes in the presence of a selective pressure for one trait. Co- and cross-resistance might explain the persistence of resistance in cases where the actual antimicrobial has not been used for a longer period of time. This phenomenon is well-known in veterinary medicine. For example, swine *E. coli* isolates have been reported resistant to chloramphenicol in spite of the absence of a direct selection pressure exerted by chloramphenicol use for more than 25 years, as this product was withdrawn from the

market for food producing animals in 1989 in Europe (Callens et al., 2012). Cross-resistance to florfenicol and co-resistance by the use of aminoglycosides, tetracyclines and sulfonamides may explain this persistent chloramphenicol resistance (Bischoff et al., 2005). Cefazolin-resistant *E. coli* strains, harboring extended spectrum class A or class C β-lactamases on plasmids, have been isolated from broiler chickens in Japan (Kojima et al., 2005). Since no cephalosporins have been approved for use in poultry in Japan, the selection of these strains might have been enhanced by the presence of other resistance genes on the same plasmid (Harada and Asai, 2010) or by the use of other β-lactam antibiotics also inactivated by these β-lactamases. Co-selection of *strA* and *sul2* genes, conferring resistance to streptomycin and sulfonamides respectively, has been reported during the treatment of chickens with streptomycin (Faldynova et al., 2013). Co-selection is not limited to the use of antimicrobials (Pal et al., 2015b). Resistance genes can be linked to a much broader spectrum of genes due to common mobile genetic elements, such as plasmids, transposons or Insertion Sequences (IS). Integrons represent also an important mechanism for the acquisition of resistance genes in many bacteria and while integrons as such are not autonomously mobile, they become 'mobile' once they are coupled with mobile DNA elements *e.g.* IS, transposons. These resistance genes can attribute an advantage to bacteria in certain conditions, such as in the presence of heavy metals (Baker-Austin et al., 2006; Cavaco et al., 2011; Pal et al., 2015), biocides (Levy, 2002; Pal et al., 2015), nutritional components in a diet (Khachatryan et al., 2006) and immune defense mechanisms (Goswami et al., 2008). As a result, selection of resistance genes can occur by other selectors, even in the absence of an antimicrobial selection pressure. Specific efflux and multidrug efflux systems for antimicrobials can for instance be involved in additional physiological functions related to a wide range of potentially toxic

substances occasionally including also antimicrobial agents (Butaye et al., 2003; Wang et al., 2000). This may confer advantages to bacteria even when antimicrobials are not present, resulting in the persistence of such systems. The effects of non-antimicrobial factors on selection and spread of AR are, however, beyond the scope of the current review and will not be discussed here.

Effect of total amount of antimicrobials used

Stuart Levy introduced the threshold theory, suggesting that a certain level of antimicrobial drug consumption is required to trigger the emergence of resistance in a particular environment. This theory is based on the concept of an equilibrium between the number of susceptible and resistant bacteria and the potential of the population of susceptible bacteria to return to their original number after an antimicrobial treatment (Levy, 1994). Austin et al. (1999) supported this theory by describing the sigmoidal rise in resistance over time in the presence of a constant rate of antimicrobial consumption. Again, this idea suggests that a critical level of drug consumption is required to trigger the increase of resistance to certain levels. This also implies that small changes in the volumes of antimicrobials, used in a population with a low level of AR, may lead to much larger changes in resistance when compared to the effect of comparable changes in use in a population where already a high level of resistance is present (Austin et al., 1999; Handel et al., 2006). This highlights the importance of reacting on emerging resistance at the earliest possible phase.

In vivo studies in cattle (O'Connor et al., 2002) and pigs (Dunlop et al., 1998b), aiming to evaluate the effect of different administration routes on the selection and spread of resistance, observed that in animals already receiving an in-feed antimicrobial, no further increase of the resistance prevalence was observed after an additional subcutaneous administration of the same antimicrobial. Resistance prevalence to

antimicrobials not present in the feed increased after administering them via subcutaneous injection. Berge et al. (2006) equally observed no resistance increase in high level resistant *E. coli* from pre-weaned calves after individual antimicrobial treatment concurrently with antimicrobials administered in the milk replacer. Comparing other groups (Berge et al., 2006), calves not receiving in-milk antimicrobials but treated individually transiently shed a more resistant *E. coli* population than untreated calves. This again suggests that the in-feed antimicrobials might have increased the level of resistance to a saturation level and that additional treatments did not result in a further increase of the prevalence of resistance (Dunlop et al., 1998a; O'Connor et al., 2002). This so called “saturation level” appears to be different for different antimicrobial agents and different types of resistance. The factors affecting it are not yet fully understood.

The non-linearity of the association between use and resistance may partially explain the sometimes observed weak or even apparent absence of a link between antimicrobial use and resistance selection (Checkley et al., 2008). Moreover, one needs to take into account that, when studying the link between use and resistance in bacteria based upon field data, the observed levels of resistance are a reflection of the current and historical use, whereas the measured use often only reflects the recent use or in the best case a retrospect of only a short period.

Effect of antimicrobial dose and duration of treatment

Appropriate dosage regimens should aim for the highest microbiological and clinical efficacy of a treatment and for the lowest selection of resistance (Roberts et al., 2008) both in the targeted pathogens and the commensal bacteria. This requires a good understanding of resistance mechanisms involved as well as knowledge of the pharmacodynamics and kinetics of the antimicrobials used. However, diverging results

between *in vitro* and *in vivo* studies on the impact of different dosage regimens on resistance selection and spread exist (Roberts et al., 2008; Smith et al., 2003). The majority of the studies describing the relationship between dosing and resistance have focused on mutational resistance mechanisms (Smith et al., 2003) and on the effect of under-dosing in resistance selection. Studies focusing on duration of treatment or linking antimicrobial dose with horizontal transfer of resistance are limited.

Mutational resistance selection and antimicrobial dose

The appropriate dosage regimens are diverse for different antimicrobial classes and bacterial species. In the current literature, the relationship between the dosage on the one hand and selection and spread of AR on the other hand is well illustrated for many fluoroquinolones, where the evaluated resistance mechanisms were in most cases the same *in vitro* as in the clinical setting (Smith et al., 2003). This allows determining the dosage that limits the selection of resistant mutants, also referred to as the “mutation prevention concentration” (MPC). Tam et al. (2005) demonstrated the possibility of combining optimal treatment dosages and the suppression of resistance emergence for garenoxalin resistance in *Pseudomonas aeruginosa* in an *in vitro* model. The inverted “U” shape relationship between exposure and resistance selection (Fig. 2) indicated that a range of antimicrobial concentrations might favor isolates with higher MICs and cause a considerable amplification of the resistant subpopulation (Baquero and Negri, 1997; Tam et al., 2007). This MPC concept has encouraged the use of high dose regimens to reduce the likelihood of selection of resistant mutants (Lubbers et al., 2011).

Confirmatory findings were seen for enrofloxacin treatments in dogs (Awji et al., 2012) and grass carp (Xu et al., 2013), where only the highest doses within the clinically

recommended dose ranges could achieve sufficient high concentrations to cross the MPC (Awji et al., 2012) and therefore limit selection for resistant mutants.

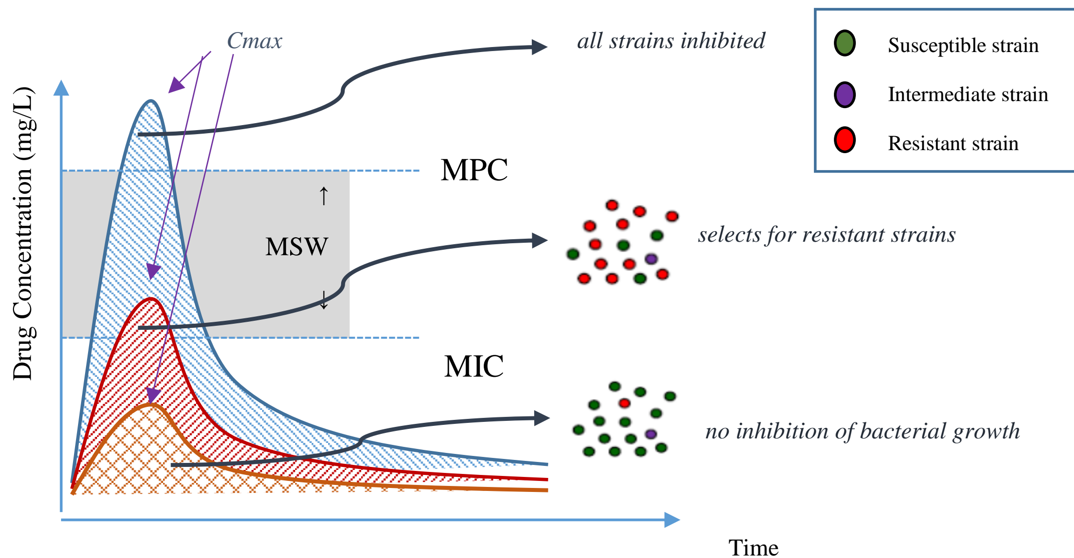

Figure 2. Curves represent the pharmacokinetics of an antimicrobial agent. The area under the curve (AUC) is depicted for 3 different treatment doses. If the drug's peak serum concentration (Cmax) does not reach the minimum inhibitory concentration (MIC) it will not inhibit the growth of any strain (orange curve). However, if the drug's Cmax lies within the mutant selection window (MSW, this is the concentration between the MIC and MPC) it selectively amplifies the resistant subpopulation (red curve). When the drug's Cmax exceeds the mutant prevention concentration (MPC) it effectively inhibits both susceptible, intermediate and first-step resistant strains (blue curve). (modified with permission by Canton, 2011)

In vitro studies revealed that the MPC of the combination of a macrolide and rifampin against *Rhodococcus equi* was considerably lower than when the individual antibiotics were tested. Antimicrobial concentrations obtained in lung tissue of treated foals are higher than this MPC, indicating that combining a macrolide with rifampin may considerably decrease the emergence of resistant *R. equi* mutants (Berghaus et al., 2013).

Several authors have, however, questioned the MPC concept and the derived dosage regimens for preventing the emergence of AR, as this concept suggests that there is no selection for mutants at concentrations lower than the MIC (Canton and Morosini,

2011; Courvalin, 2008; Macia et al., 2011). This is contradictory to observations that concentrations below MIC may facilitate hypermutation and horizontal gene transfer (Canton and Morosini, 2011; Macia et al., 2011). Furthermore, resistant mutants may have benefits compared to the susceptible strains at sub-MIC levels, as long as their fitness cost is lower than the growth reduction of the susceptible isolates (Gullberg et al., 2011).

The use of specific (e.g. single, high dose) dosing regimens as a potential way of reducing resistance emergence might be restricted to specific antimicrobial-bacterial relations. Even if the correct dosing regimen is known and applied in the field, at the same moment the commensal microbiota of the animal, for which the treatment dose is not specifically adapted, is also exposed to these treatment doses. Also, a correct dosing for a specific pathogen in a specific organ may be an over- or under-dosing for a commensal present in the same or another organ (Devreese et al., 2014).

Under-dosing and selection of AR

The use of antimicrobial growth promoters in livestock is clearly in contrast with the concept of the establishment of single and high treatment dose regimens, as growth promoters can be regarded as under-dosed antimicrobial compounds. Various studies have linked the use of antimicrobial growth promoters to the occurrence of AR among Gram-positive and Gram-negative commensal bacteria (Alexander et al., 2008; Sunde et al., 1998; van den Bogaard and Stobberingh, 2000). For commensal and pathogenic bacteria, not only mutational resistance is of concern, but especially the organization of multi-drug resistance clusters as demonstrated e.g. in *Enterobacteriaceae* (Leverstein-van Hall et al., 2002). This type of resistance is frequently encoded by genes located on mobile genetic elements, such as plasmids or transposons. Feeding sub-therapeutic concentrations of tylosin to pigs on a continuous basis (Holman and

Chenier, 2013) or chlorotetracycline with sulfamethazine to cattle feedlot (Alexander et al., 2008) led to the acquisition of such resistance genes selecting for isolates with 100-fold increased MICs.

In contrast, several studies in swine *E. coli* (Langlois et al., 1984; Wagner et al., 2008), *Salmonella enterica* (*S. enterica*) (Wagner et al., 2008) and anaerobes (Holman and Chenier, 2013) after administering different dosages of tetracycline did not confirm the statement that underdosing would lead to a higher degree of resistance in comparison to correct dosing. Moreover, Langlois et al. (1984) found a significantly higher resistance level in the group receiving the higher dosage compared to the group receiving the lower dosage during the first weeks after administration. This turned to the opposite from 30 days after first administration to the last sampling at 84 days, suggesting the time period after administration also needs to be taken into consideration.

Cross-contamination of plain feed with medicated feed is a relatively neglected case of under-dosing (Filippitzi et al., 2016; Peeters et al., 2016). Yet, also when very low concentrations of antibiotics (e.g. 1mg/L doxycycline) were administered to pigs, a selection towards AR was seen (Brewer et al., 2013; Peeters et al. 2017 (in press)).

Effect of the type of antimicrobial agent chosen for treatment

Antimicrobial agents are characterized by several features that may play a role in the selection and spread of resistance. In addition to differences in mode of action of the antimicrobials and their spectrum of susceptible bacteria and whether they exert bacteriostatic or bactericidal effects, they may have diverging pharmacodynamic and kinetic parameters. These include differences in plasma half-life, tissue distribution and tissue persistence, which are important for their time- or concentration-dependent activity.

The use of antimicrobial agents with a broader spectrum affects a higher number of different bacterial taxa and thereby may increase the risk for selection of bacteria carrying resistance genes compared with agents with a narrower spectrum. Also, it may increase the risk for suppressing or eliminating broadly the susceptible commensal microbiota, which generally outcompetes resistant strains (Levy and Marshall, 2004). Thus, these broad-spectrum agents might encourage the survival of more resistant strains. However, the distinction between broad-and narrow-spectrum is not always that straightforward as often stated. For instance, benzyl penicillins are usually classified as antibiotics with a Gram-positive spectrum, although they are also active against many Gram-negative bacteria, including *Pasteurellaceae* and most Gram-negative anaerobes. Bacteriostatic antibiotics might select more for resistant sub-populations than bactericidal ones, because they only inhibit growth and do not kill the bacteria (Dagan et al., 2001). Yet, bactericidal antibiotics might eradicate fully susceptible populations giving the opportunity for resistant strains to colonize certain ecological niches (Catry et al., 2008). The distinction between bactericidal and bacteriostatic effect is far from being absolute and depends on both the drug concentration at the site of infection and the bacterial species involved (Prescott and

Dowling, 2013). Sub-lethal antimicrobial concentrations can induce stress in the targeted bacteria which may favor mutations (Canton and Morosini, 2011; Macia et al., 2011) and might also result in a transient decrease in antimicrobial susceptibility due to increased copy numbers of resistance genes (McMahon et al., 2007).

Different antimicrobials will also select for different levels of resistance genes expression. Resistance to macrolides, lincosamides and streptogramin B (MLS_B resistance) encoded by *erm* genes can be either constitutive (permanently expressed) or inducible (expressed after antimicrobial exposure). This inducible resistance can have clinical implications as *in vivo* exposure to macrolides may result in resistance higher than predicted by *in vitro* determined MICs in the absence of the inducer (Chancey et al., 2011). In staphylococci, within the macrolides class, only the 14- (e.g. clarithromycin and erythromycin) and 15- (e.g. azithromycin) member rings are good inducers for resistance expression (Chancey et al., 2012). Thus, isolates harboring inducible MLS_B resistance and exposed to 16-membered (e.g. tylosin) antimicrobials can remain susceptible, whereas constitutive MLS_B resistance refers to all macrolide members.

For time-dependent antimicrobials, such as β-lactams, tetracyclines, macrolides, sulfonamides and lincosamides, the antibacterial effect is highest when the concentration is maintained above the MIC throughout the dosing interval. Long-acting formulations, based on long half-lives, result in prolonged plasma concentrations in tissues, and offer a solution for the required repeated administrations inherent to treatment regimens of time-dependent antimicrobials. Such long-acting formulations have been developed for certain third-generation cephalosporins (for example cefovecin) or macrolides (azithromycin, tulathromycin). They are characterized by a long half-life and a slow release after tissue binding, resulting in a pronounced post-

treatment effect (Van Bambeke and Tulkens, 2001). For azithromycin, this effect has been shown to significantly select more for macrolide-resistant streptococci until about 4 weeks after the end of therapy than clarithromycin, characterized by shorter plasma half-life and tissue persistence (Malhotra-Kumar et al., 2007). Moreover, concentrations of macrolides below the MIC and long-term presence due to a long half-life, can favor mutational resistance, as has been shown *in vitro* for *S. pneumoniae* (Nagai et al., 2000; Pankuch et al., 1998).

In conclusion, the choice of a specific antimicrobial should not be based exclusively on the elimination of the target pathogen, but should equally take into account minimizing the selection of resistance determinants.

Effect of the administration route

Different factors might play a role in the effect of the administration route on resistance selection and spread. At first, the route of administration will affect tissue and intestinal content concentrations (Baggot and Giguère, 2013) and thus also the degree of the selection pressure exerted on both pathogens and commensal bacteria in different organ systems. Oral administration of antimicrobials exerts a selection pressure on the intestinal microbiota that is most likely higher than seen for parenteral injections, except for parenterally administered antimicrobials which undergo enterohepatic circulation to a high extent, such as tetracyclines (Prescott and Dowling, 2013). The degree of absorption of antibiotics in the upper gastrointestinal tract after oral administration may also influence selection pressure exerted on the microbiota of the large intestine. Systemic availability of amoxicillin is about twice that of ampicillin after oral administration (Prescott, 2013). Therefore, ampicillin may exert more selection pressure in the lower intestinal tract than amoxicillin.

In food producing animals individual treatments are mostly parenteral and group treatments are mostly oral (Callens et al., 2012; Pardon et al., 2012). Treatment of only one or a few animals (individual treatment) compared to an entire group of animals can affect observed resistance levels. However, resistance selection, as the result of antimicrobial treatment of a single animal, may be partially diluted at the population level due to the presence of a susceptible microbiota excreted by the contact animals. In chickens, previously fed tetracycline-containing feed, a decrease in the excretion of resistant *E. coli* was seen after housing them with larger numbers of cage mates that excreted susceptible microbiota (Levy, 1978). However, when the entire population is treated, the odds of dilution to occur by susceptible bacteria will be lower and a commensal reservoir of resistance genes can be formed (Levy and Marshall, 2004). Moreover, the transfer of resistant bacteria might occur more rapidly to animals being treated, due to the disturbance of the commensal microbiota, which exerts a protective effect against colonization and infection by exogenous organisms (Barza and Travers, 2002). Dunlop et al. (1998a) compared the effect of individual and group treatment on resistance in *E. coli* from swine using aminoglycosides and tetracycline and found lower resistance levels in the group receiving individual parenteral treatment compared to the group receiving oral administration. Feedlot bulls showed a higher proportion of resistant *E. coli* after the oral administration of tetracyclines compared to a subcutaneous treatment (Checkley et al., 2010), yet the prominent difference disappeared after a few weeks, showing that other factors were involved as well. The authors suggested an exchange of bacteria between the groups, as all bulls were kept together. On the one hand, a dilution effect could have occurred in the animals treated with feed antimicrobials after the antimicrobial selection pressure dropped. On the other hand, resistance might have spread horizontally between the different groups of

animals, explaining the rise in resistance after cessation of antimicrobial therapy in both the control and the parenterally-treated group.

Regarding administration route, oral treatment seems to exert a higher selection pressure on intestinal bacteria than parenteral treatment although different factors might play a role. Yet, only limited specific research data on the effect of different administration routes on resistance selection and spread are available. The development of resistance in intestinal bacteria of mice was significantly less or delayed when the same doses of antimicrobials were administered via intravenous injection rather than oral administration (Zhang et al., 2013). Moreover, the difference in intravenous or oral therapy was more significant for ampicillin, eliminated via the kidney, than for tetracycline, excreted via both kidneys and the gastrointestinal tract. Wiuff et al. (2003) included a parenteral and an oral group treatment in their study and found no difference in the speed of selection for resistance in *S. enterica* infected pigs between intramuscular administration of enrofloxacin and oral administration of the same dose. Yet, a selection pressure might also have been present in the intestines following parenteral administration, as enrofloxacin and its major metabolite, ciprofloxacin, is passing through the intestinal tract after excretion in the bile in humans (Koningstein et al., 2010). From these studies, it appears that the effect of different administration routes on resistance selection again depends on the antimicrobial used, as this is linked to a specific excretion route.

Conclusions

It can be concluded that the total amount of antimicrobials used, the dosing regimen, the type of antimicrobial agent, and the administration route -all factors related to antimicrobial usage- influence the selection, spread and persistence of AR in farm animals. The complex interaction between these factors complicates interpretation of

their effects. In the fight against AR, these factors should be taken into account. Avoiding unnecessary antibiotic use should be the priority. Whenever antibiotics are required, treatment strategy should be carefully planned. The choice of a specific antimicrobial should not be based exclusively on the elimination of the target pathogen, but should equally take into account all aspects aiming at a minimal selection of resistance determinants both in pathogenic and commensal bacteria.

Table 1. Factors relating to the use of antimicrobials and their effects on antimicrobial resistance selection and spread

Antimicrobial factors	References	Study set up	Microorganism	Host	Antimicrobial (AM) studied	Study focus
Total amount of antimicrobials used	Levy, 1994	Opinion paper	NS[1]	Human	NS	Selection threshold level different for different AM[2]s
	Austin, 1999	Mathematical (predictive) model	NS	Human	NS	Sigmoid rise in AR[3] over time when an AM is administered at a constant rate
	Handel, 2006	Mathematical (predictive) model	*Neisseria gonorrhoeae*	Human	NS	Small changes in levels of treatment can lead to large changes in resistance emergence
	O'Connor, 2002	*In vivo*	*Escherichia coli*	Bovine	Oxytetracycline, chlortetracycline	Higher AR ratios when AMs administered orally
	Dunlop, 1998a, b	*In vivo*	*E.coli*	Porcine	Tetracycline	Differences between individual and group treatments
	Berge, 2006	*In vivo*	*E. coli*	Calves	Neomycin, tetracycline	Prophylactic vs therapeutic AM administration
Dose and duration	Roberts, 2008	Review	NS	Human	Fluoroquinolones, aminoglycosides, carbapenems, β-lactams, glycopeptides	AR and dosing effect
	Smith, 2003	Opinion paper	NS	NS	Aminoglycosides, β-lactams, macrolides, fluoroquinolones	Applicability of MPC and its limits
	Tam, 2005	*In vitro*, mathematical modelling	*Pseudomonas aeruginosa*	NS	Garenoxacin	Applicability of MPC. Bacterial population responses to drug selective pressure
	Baquero and Negri, 1997	Review	NS	NS	NS	Treatment dose and duration : effects on the selective activity of an antibiotic regimen
	Tam, 2007	*In vitro,* mathematical modelling	*Staphylococcus aureus*		Ciprofloxacin, garenoxacin	Therapy duration is a critical parameter on the emergence of fluoroquinolone resistance
	Lubbers, 2011	Simulation study, *in vitro*	Donor: *Salmonella enterica* serovar Typhimurium. Recipient: *E. coli*	Bovine	Oxytetracycline	Role of AR plasmids

Awji, 2012	*in vivo*	*Staphylococcus pseudointermedius*	Dog	Fluoroquinolones	Role of MPC
Xu, 2013	*In vivo*	*Aeromonas hydrophila*	Grass carp	Enrofloxacin	Role of MSW MPC, PK/PD, pharmacotherapy for prevention of resistant strains
Blondeau, 2012	*In vitro*	*Mannheimia haemolytica*	Bovine	Enrofloxacin, ceftiofur, florfenicol, tilmicosin, tulathromycin	Role of MIC, MPC
Berghaus, 2013	*In vitro*	*Rhodococcus equi*	Equine (foals)	Erythromycin, clarithromycin, azithromycin, rifampin, amikacin, gentamicin, enrofloxacin, vancomycin, imipenem and doxycycline	Role of MSW[6] , MPC
Almeida, 2007	*In vivo*	*Mycobacterium tuberculosis*	Mice	Moxifloxacin	Role of MSW, MPC
Cui, 2006	*In vivo*	*S. aureus*	Rabbit	Levofloxacin	Role of MPC, MSW
Canton and Morosini, 2011	Review	NS	NS	Fluoroquinolones	Emergence spread of AR following exposure to AM's / Focus on MPC MSW
Courvalin, 2008	Opinion paper	NS	NS	Fluoroquinolones	Role of MPC, MSW
Macia, 2011	*In vitro*	*P. aeruginosa*	Human	Ciprofloxacin	Effect of biofilm growth, AR and mutator phenotypes, MPC
Gullberg, 2011	*In vitro*	*E. coli, S. enterica* serovar Typhimurium	NS	Tetracyclines, fluoroquinolones, aminoglycosides	Selection of resistance due to very low antibiotic concentrations
Devreese, 2014	*In vivo*	NS	Chicken	Enrofloxacin, ciprofloxacin	Effect of administration route and dose on plasma and intestinal concentrations
Alexander, 2008	*In vivo*	*E. coli*	Cattle	Chlortetracycline plus sulfamethazine, virginiamycin, monensin, tylosin	Role MICs and AR
Sunde, 1998	*In vivo*	*E. coli*	Porcine	Sulfonamides, trimethoprim, streptomycin, ampicillin, neomycin, chloramphenicol, tetracycline	Role of commensal E.coli as a considerable reservoir of AR genes
van den Bogaard and Stobbering, 2000	Opinion paper	*E. coli*	Human, farm animals	NS	Reduction of AM use
Leverstein-Van Hall 2002	*In vivo*	Enterobacteriaceae	Human	NS	Role of integrons, focus on MDR[7] strains
Holman and Chenier, 2013	*In vivo*	Total microbiome	Porcine	Chlortetracycline, tylosin	Sub-therapeutic dosing effects
Langlois, 1984	*In vivo*	*E. coli*	Porcine	Chlortetracycline	Effect of sub-therapeutic and therapeutic dosage in AR
Wagner, 2008	*In vivo*	*S. enterica, E. coli*	Porcine	Chlortetracycline, tylosin	Effect of dosing regimens
Brewer, 2013	*In vivo*	*E. coli, S. enterica* serovar Typhimurium, *Yersinia enterocolitica, Shigella flexneri, Proteus miralbilis*	Porcine	Apramycin, lincomycin, neomycin, florfenicol, hygromycin, penicillin G, roxarsone, sulfamethazine, tetracycline, tylosin	Sub-therapeutic dosing effects
Peeters, 2017	*In vivo*	*E. coli*	Porcine	Doxyxycline	Residual effect of doxycycline on the selection of doxycycline resistance
Peeters, 2016	*In vivo*	-	Porcine	Chlorotetracycline, doxycycline, sulfadiazine-trimethoprim	Quantitation of antimicrobial residues in cross-contaminated conventional animal feed
Filippitzi, 2016	Risk model	-		Sulfonamides, penicillins, tetracyclines, macrolides, polymixins	Cross-contamination risk due to use of medicated feed

Choice of antibiotics	Levy and Marshall 2004	Review	-	Human	NS	AR and effect of AM with a narrow or broad spectrum
	Dagan, 2001	Review paper	*Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis*	NS	NS	Bacteriostatic agents selecting for resistant subpopulations
	Catry, 2005	*In vivo*	*Pasteurella multocida, Trueperella pyogenes*	Calves	Fluoroquinolones	Treatment selected for resistant strains to colonize the upper respiratory tract
	Prescott and Dowling, 2013	Book chapter	*NS*	NS	NS	Distinction between bacteriostatic and bactericidal AMs is not clear
	McMahon, 2007	*In vitro*	*E. coli, Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*	NS	Applied sub-lethal stress	Use of bacteriostatic AMs contribute to the development and spread of AR
	Chancey 2011, Chancey 2012	*In vitro*	*S. pneumoniae*	NS	Macrolides	14-, 15-member rings are good inducers for resistance expression
	Van Bambeke and Tulkens, 2001	Review	NS	NS	Macrolides	Long-acting formulations can result in a pronounced post-treatment effect
	Malhotra-Kumar, 2007	*In vivo*	Streptococci	Human	Azithromycin, clarithromycin	Role of *ermB* resistance gene
	Nagai, 2000	*In vitro*	*S. pneumoniae*	-	Ceftriaxone, cefprozil, azithromycin	Ceftriaxone selected for AR less often than cefprozil and azithromycin
	Pankuch, 1998	*In vitro*	*S. pneumoniae*	Human	β-lactams, azithromycin	Role of *mefA, ermB* resistance genes
Administration route	Baggot and Giguère, 2013	Book Chapter	NS	Animals	All AM classes	Principles of antimicrobial drug bioavailability and disposition
	Prescott and Dowling, 2013	Book Chapter	NS	Farm animals	Tetracyclines	Tetracycline mechanism of action and main resistant mechanisms
	Prescott, 2013	Book Chapter	NS	Animals	Ampicillin and amoxicillin	Systemic availability of amoxicillin and ampicillin
	Callens 2012	*In vivo*	NS	Porcine	All common AMs	Injectable products were overdosed, whereas oral treatments often underdosed
	Pardon 2012	*In vivo*	NS	Veal calves	Enrofloxacin, flumequine, amoxicillin, ampicillin, tylosin, tilmicosin, oxytetracycline, doxycycline, TMP/sulphonamides, colistin	Oral treatment is predominantly used, under-dosing is common
	Levy, 1978	*In vivo*	*E. coli , P. mirabilis,* enterococci	Chicken, Human	Oxytetracycline	Emergence of AR bacteria, oral administration (feed)
	Levy and Marshall 2004	Review	-	Human	NS	AR and effect of entire population treatment vs individual treatment
	Barza and Travers, 2002	Meta-analysis, risk assessment	*S. enterica., C. jejuni*	Human	NS	Excess infections due to AR. Transfer of resistant bacteria more rapidly to treated patients.
	Dunlop, 1998	*In vivo*	*E. coli*	Porcine	Ampicillin, gentamicin, nitrofurantoin, spectinomycin, sulfisoxazole, tetracycline	Treatment schemes and AR patterns associations

Checkley, 2010	*In vivo,* clinical trial	*E. coli*	Cattle	Oxytetracycline	More resistant strains after oral treatment than after subcutaneous treatment
Zhang, 2013	*In vivo*	*Enterococcus* spp., bla_{CMY}-carrying *E. coli*	Mice	Tetracycline hydrochloride, ampicillin sodium	Oral vs *i.v.* injection and different doses
Wiuff, 2003	*In vivo*	*S. enterica* serovar typhimurium*, E. coli*	Porcine	Enrofloxacin	Parenteral (*i.m.)*, oral and different doses
Koningstein, 2010	Case- control study	*Salmonella* spp.	Human	Fluoroquinolones, tetracyclines, sulphonamides, trimethoprim and broad-spectrum penicillines	Prior use of fluoroquinolones increases the risk of fluoroquinolone-resistance in *Salmonella* infections

[1]: Not specified, [2]: antimicrobial, [3]: antimicrobial resistance, [4]: minimal inhibition concentration, [5]: mutant prevention concentration, [6]: mutant selection window, [7]: multi-drug resistant

Table 2. Co-selection, co-resistance and cross-resistance principles

Term	Definition	Mechanisms and Examples	Comments
Co-selection	The selection (*via* acquisition or overexpression) of one or more resistance gene(s) that confer(s) resistance to additional agents as a result of genetic linkages or physiological adaptations.	• Co-resistance (see below) • Cross-resistance (see below) • Co-regulation (e.g. zinc administration led to upregulation of *mdtABC* operon in *E. coli* resulting in resistance against novobiocin and deoxycholate[3]) • Biofilm induction as a mode of co-selection (e.g. role of 'persister' cells[4])	Clones and clonal complexes are units for AR selection[1] Ecological factors are to be considered as well[2]
Co-resistance	Involves transfer of several genetic elements into the same bacterial isolate and/or the acquisition of mutations in different genetic loci affecting different antibacterial classes of drugs	○ Plasmid (e.g. co-located on a single plasmid there is *tcrB* gene (conferring resistance against copper resistance), *vanA* and *ermB* genes (conferring resistance against macrolides[9])) ○ Transposon (e.g. Tn 21 containing *mer* genes that confer resistance against Hg and carrying blaCTX-M[10,11]) ○ Integron (e.g. Class 1 integron contains both *qac* that confers resistance against quaternary ammonium and *sul1* that confers resistance against sulphonamides)	Consequence of genetic linkages
Cross-resistance	A single resistance mechanism conferring resistance to several antibiotics of the same class (e.g. aminoglycoside-modifying enzymes) or across different classes of antibiotics (e.g. MDR pumps in *Listeria monocytogenes*)	▪ Efflux-pump upregulation (e.g. *AcrAB-TolC* in *E. coli*[5] conferring resistance against fluoroquinolones, tetracycline and chloramphenicol) ▪ Over-expression (e.g. ABC transporters in *E.coli* conferring resistance against amino acids, ions, sugars, lipids and drugs[6]) ▪ Reduced cell envelope permeability (e.g. *pmr* gene in *E. coli* conferring resistance against polymyxin B and aminoglycosides[7]) ▪ Alteration in a target site or acquisition of a neutralizing enzyme (e.g. AAC(6′)-Ib-cr enzyme in *E. coli* conferring resistance against various aminoglycosides and fluoroquinolones[8])	Consequence of physiological adaptations

[1]: Canton and Ruiz-Garbajosa, 2011, [2]: Seiler and Berendonck, 2012, [3]: Baker-Austin et al., 2006, [4]: Harrison et al, 2005, [5]: Soto et al, 2013, [6]: Dawson et al, 2006, [7]: Delcour et al, 2009, [8]: Park et al, 2006, [9]: Freitas et al, 2011, [10]: Kiyono et al, 2009, [11]: Canton and Coque, 2006.

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1.2 Antimicrobial Resistance Selection Pressure And Spread In Food Producing Animals Through Factors Other Than Antimicrobial Agents Use

Antimicrobial Resistance Selection Pressure and Spread in food producing animals through Factors other than Antimicrobial Use

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Abstract

Since their discovery, antimicrobials have become indispensable tools in countering bacterial diseases in both humans and animals. Yet, their use has become overshadowed by antimicrobial resistance which has become increasingly more threatening over the last decades. The emergence of resistant bacteria has accelerated in recent years, mainly as a result of increased selective pressure through the use of antibiotics (Beceiro et al., 2013). However, besides the use of antimicrobials, other factors can be involved in the selection and spread of resistance determinants. These factors include selection pressure originating from the use of biocides or heavy metals, age and stress conditions of the hosts, animal husbandry practices, diet and bacterial related factors such as virulence and bacterial fitness. These non-antimicrobial selection factors are often ignored, even though they may play an important role in persistence of antimicrobial resistance in the presence or absence of anthropogenic antimicrobial use.

In this paper an overview is given of all important non-antimicrobial factors that may influence the selection and spread of antimicrobial resistance.

Introduction

Antimicrobial resistance is an ever-growing global concern. New bacterial resistance mechanisms are emerging and spreading globally, threatening our ability to treat bacterial diseases (WHO, 2016). Different mechanisms can lead to antimicrobial resistance such as activation of efflux pumps, target modification or replacement, reduced permeability and antimicrobial agent modification (Boerlin and White, 2013; Prescott and Dowling, 2013; Wright, 2010). Several free-living bacteria and fungi produce antimicrobial compounds as a means to compete with surrounding micro-organisms for nutrients. These natural products are defined '*antibiotics sensu stricto'* and are ubiquitously present (D'Costa et al., 2011; Waksman and Woodruff, 1940). Some resistance mechanisms are believed to have originated from such bacteria or fungi to protect themselves from the compounds they produce (Benveniste and Davies, 1973; D'Costa et al., 2011; Waksman and Woodruff, 1940). Resistance mechanisms may also have evolved from pathways involved in other physiological processes, such as detoxification of metabolic intermediates, virulence and other functions (Piddock, 2006). This might explain the ancient nature of antimicrobial resistance, existing in nature long before the presence of anthropogenic antimicrobials, defined as antimicrobials produced and used by humans (Allen et al., 2009; D'Costa et al., 2011).

Antimicrobial resistance genes can spread within a population by vertical dissemination or horizontal transmission to more or less related species through transformation, transduction or conjugation (Holmes et al., 2016). Once antimicrobial resistance has emerged, the proportion of isolates with such resistance determinants can increase as the result of a selection pressure. The major driver of selection for antimicrobial resistance is antimicrobial use (Holmes et al., 2016; Silbergeld et al., 2008). However, besides the use of antimicrobials, also other factors can be involved

in the selection and spread of resistance determinants. These non-antimicrobial selection pressures are often ignored in discussions or programs to reduce antimicrobial resistance. Since these factors can influence the spread and persistence of antimicrobial resistance in the presence or absence of anthropogenic antimicrobial use, understanding them better can support the struggle against antimicrobial resistance.

Below an overview is given of the documented non-antimicrobial factors that may influence the selection and spread of antimicrobial resistance.

Chemical factors

Biocides

Biocides encompass chemicals with antiseptic, disinfectant and/or preservative activity (McDonnell and Russell, 1999). Their use is essential to meet the hygienic needs of food animal production. Yet, intrinsic and acquired antimicrobial resistance mechanisms have been described in bacteria that confer resistance to several biocides such as quaternary ammonium compounds (QAC), triclosan, cetrimide, chlorhexidine, benzalkonium chloride (BC). The majority of these resistance mechanisms are conferring resistance against antibiotics as well (McMurry et al., 1998; Levy, 2002; Fraise, 2002). The use of these biocides may therefore select for antimicrobial resistance against both antibiotics and disinfectants. Cross-resistance between antibiotics and disinfectants may be due to a single resistance mechanism providing resistance to both antibiotics and disinfectants (Table 1). Various studies in *Escherichia coli (E. coli), Staphylococcus aureus, Pseudomonas aeruginosa (P. aeruginosa) and Salmonella enterica (S. enterica)* have demonstrated that efflux pumps play an important role in resistance to both antimicrobials and disinfectants, including QAC, cetrimide, chlorhexidine, BC and triclosan (Levy, 2002; Hegstad et al.,

2010). Induced cross-resistance following increasing concentrations of either a biocide or an antibiotic was demonstrated under laboratory conditions (Braoudaki and Hilton, 2004). Apart from that, sub-inhibitory concentrations of QAC triggered *in vitro* the overexpression of *acrAB* and led to the selection of *S. enterica* serovar Typhimurium strains resistant to QAC, ampicillin, and tetracycline (Karatzas et al., 2007). The latter is of special importance for the practice, as these sub-inhibitory concentrations have been repeatedly reported due to poor disinfection procedures (Langsrud et al., 2003; McDonnell and Russell, 1999).

Besides a common resistance mechanism, also genetic linkage between antibiotic resistance genes and genes encoding resistance to disinfectants may result in the selection for antimicrobial resistance by the use of biocides. This has been demonstrated in *Staphylococcus aureus* strains carrying plasmids with both β-lactamase genes and *qac* genes encoding QAC resistance (Fraise, 2002). In *S. enterica* and *E. coli* isolates from farm animal origin, *qac* genes can be co-located with *sul1* genes (encoding sulphonamide resistance) on type-I integrons, and may also harbor various other resistance genes (Chuanchuen et al., 2007; Cocchi et al., 2007; Sidhu et al., 2002; Sidhu et al., 2001).

A third factor, along with co- and cross-selection, might be the selective stress exerted by biocides. Overall, bacterial stress refers to the ability of bacteria to adapt to a chemical or other applied stress. It alters gene expression patterns and cell physiology in ways that can and do influence antimicrobial susceptibility (Russell, 2003). One example is the expression of the broad-specificity efflux *acrAB* pump that is well known for its ability to transport vectorially a diverse array of compounds with little chemical similarity, thus conferring resistance to a broad spectrum of antibiotics and biocides (Du et al., 2014). *AcrAB* pump is upregulated by the *mar* operon responding to toxic

substances, such as biocides (Levy, 2002). Furthermore, stress induced by biocides, may not only favor the expression of resistance mechanisms, but also their dissemination by horizontal transmission of integrons (Gillings, 2013) and Integrating Conjugative Elements (ICE's), via the 'SOS response' that typically follows DNA damage and results in stress-induced mutagenesis (Beaber et al., 2004; Galhardo et al., 2009). This means that biocide-induced stress may not only increase selection of the resistant sub-population, but might also favor the transmission of resistance determinants from the resistant towards the susceptible population (Beaber et al., 2004).

However, studies so far have failed to demonstrate a link between the continuous use of biocides in communities and an increase in antimicrobial resistance (Russell, 2004). Karatzas et al. (2007) suggested that the dissemination of strains resistant to QAC or triclosan might be low due to impaired virulence. Nevertheless, QAC-resistant staphylococci isolated from human patients with bacteremia showed a significantly higher prevalence of resistance to several antimicrobials than QAC-sensitive staphylococci, indicating an association between biocide and antimicrobial resistance (Sidhu et al., 2002).

Despite evidence for associations between biocide use and selection and spread of antimicrobial resistance through the above mentioned mechanisms, data related to the occurrence of bacterial resistance following exposure to biocides in the veterinary field are scarce. The correct use of biocides for biosecurity measures in animal husbandry as a part of disease prevention to avoid the need for antimicrobials is strongly arguing in favor of biocide use (Frentzel et al., 2013) Nevertheless, an efficient cleaning step is a prerequisite to allow for an efficient disinfection afterwards as it will limit the number of bacteria in contact with (sub)inhibitory biocide concentration The limited field data

available, indicate that there is a need for further studies to elucidate the potential interaction between the (correct) use of biocides in animal facilities and the emergence of antimicrobial resistance.

Heavy metals

Metal-containing compounds are widely used as feed supplements, both to address metabolic needs and for the prevention of gastro-intestinal diseases in food-producing animals (Cavaco et al., 2011) (Table 1). Their electrostatic properties stabilize substrates or reaction intermediates in the active sites of enzymes, while their heightened reactivity is harnessed for catalysis making them essential for bacteria. However, the latter property renders several heavy metals toxic at high concentrations to bacteria (Hood and Skaar, 2012). Multidrug efflux systems that may play a role in antibiotic resistance, have been shown to be important mechanisms of resistance against heavy metals as well, in several bacterial genera (Delmar et al., 2014). Cross-resistance has for instance been described in *Listeria monocytogenes* by means of a multiple-drug resistance pump exporting metals in addition to antimicrobials (Mata et al., 2000).

An association between copper resistance on the one hand and glycopeptide and macrolide resistance on the other hand in *Enterococcus faecium* isolates has been observed in pigs, but not in broilers, calves and sheep in Denmark (Hasman and Aarestrup, 2005). This might be partly due to higher copper exposure in pigs through feed additives compared to other livestock. Most likely, this has resulted in the co-selection of the *tcrB* , *vanA* (located in Tn1546) and the *erm*(B) gene, responsible for copper , glycopeptide and macrolide resistance respectively, as they are closely located to each other on a conjugative plasmid (Hasman and Aarestrup, 2005; Mata et al., 2000).

Genes encoding metal-resistance have been found in various SCC mec cassettes (mobile genetic elements harbouring the *mecA* or *mecC* gene, responsible for methicillin-resistance) in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains (Li et al., 2011).The *czrC* gene, conferring resistance to zinc and cadmium in *S. aureus*, was found to be located within the staphylococcal cassette chromosome *mec* type V clonal complex, prevalent in methicillin-resistant *Staphylococcus aureus* (MRSA) from pigs and veal calves (Cavaco et al., 2010; Argudín et al., 2016; Cavaco et al., 2011; Li et al., 2011). However, especially in pigs where zinc oxide is of critical importance, there are conflicting views and results on the effects of zinc oxide use in the emergence and the selection of MRSA in pigs (Burch, 2014; Moodley et al., 2011; Moodley et al., 2014). Recent *in vivo* studies by Amachawadi *et al.* (2015) and Slifiertz *et al.* (2015a, 2015b) have showed that the supplementation of pig feed with high levels of zinc oxide (high concentration of zinc) can increase the prevalence and persistence of MRSA in pigs.

In multidrug resistant *Salmonella* serotypes from swine, associations were found between ampicillin, streptomycin, tetracycline and kanamycin resistance and the *pcoA* gene, conferring resistance to copper (Medardus et al., 2014). This was also the case for resistance against ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline on the one hand and the *czcD* gene, conferring resistance to zinc on the other hand (Medardus et al., 2014).

A special mention should be made for zinc oxide as its use in pig nutrition is of critical importance to combat post weaning diarrhea in pigs. Besides the aforementioned effects on MRSA, Vahjen *et al.* (2015) showed that high doses of dietary zinc oxide can increase the number of *tetA* and *sul1* genes in Gram-negative bacteria in the gastrointesinal tract. Since the modes of action of these antimicrobial resistance genes

differ (active mode for *tetA* and passive mode for *sul1*), the authors suggested that multiple mechanisms may be involved in the development of resistance. A cross-resistance mechanism may apply for *tetA,* while a co-resistance mechanism may apply for *sul1* (Vahjen et al., 2015).

Animal husbandry factors

Animal factors

Animals experiencing stress can show increased and prolonged shedding of bacteria (Rostagno, 2009), which might also promote the spread of resistance in a population of animals or into the environment (Sorum and Sunde, 2001; Verbrugghe et al., 2012) (Table 2). Heat stress was confirmed to play a role in the increase of antimicrobial resistance levels of *E. coli* to various agents in a study using pigs that were not treated with antimicrobial agents for the past ten years (Moro et al., 2000). Apart from that, increased apramycin resistant *E. coli* prevalence was observed in pigs exposed to cold and overcrowding stress (Mathew et al., 2003). Another example of a possible stress-associated resistance effect was seen in a study on the effect of florfenicol injection in steers on multi-resistance in fecal *E. coli* where cattle were rounded up from two pastures and transferred to a research institute (Berge et al., 2005b). When assessing the effect of a single dose florfenicol treatment in these feedlot cattle, higher levels of multi-resistance and prolonged resistance were seen in steers that were held in a dirt lot for one month prior to shipment to the research institute compared to steers that were gathered up directly from pasture and shipped to the research institute.

Several studies suggest that increasing age is linked with a decreased prevalence of resistant *E. coli* in dairy cattle (Berge et al., 2010; Berge et al., 2005a; Khachatryan et al., 2004; Sato et al., 2005) and in coliforms from pigs (Akwar et al., 2008; Dewulf et

al., 2007; Langlois et al., 1988). This age-related decreasing resistance prevalence is not entirely due to lowering chance of antimicrobial exposure as the animals get older, since higher levels of resistance were also noted in young pre-weaned calves that had not been previously exposed to antimicrobials compared to adult animals (Berge et al., 2010). For example, Walk et al. (2007) suggested that the fitness cost of resistance in intestinal bacteria increases as the host gastrointestinal tract matures and as competition with other microbes increases (Walk et al., 2007). Recently, it has been shown that not only is administration of antimicrobial agents to sows during lactation a risk factor for the persistence of resistant *E. coli* for their newborn offsprings but administration of an antimicrobial agent (enrofloxacin) was a risk factor for the sows at weaning as well (Callens et al., 2015). In poultry, the prevalence of resistance for multiple agents in enterococci was significantly higher in 42 day-old broilers (the typical life span of a broiler) compared to older (1-3 years old) laying hens (van den Bogaard et al., 2002). However, this difference can at least partly be explained by the different production types for broilers and laying hens. In general, antimicrobial usage is higher in broiler production than in laying hen production systems (van den Bogaard et al., 2002). Such farm factors are further described below.

Farm factors

Farm types or housing conditions have been identified as significant factors in the prevalence of resistance in different animal sectors (Table 2). These differences have often been assigned to divergent antimicrobial use between different types of farms. For instance, when comparing the results from two studies from Berge et al., phenotypic resistance to more antimicrobials was found in *E. coli* isolated from calves from calf ranches (Berge et al., 2005b) than from calves from dairy farms (2005a). In a later study by Berge et al. (2010) the degree of the multiple antimicrobial resistance (MAR) in the *E. coli* flora was measured in beef cow-calf operations and dairies (lowest MAR), feedlots (intermediate MAR) and calf ranches (highest MAR). Apart from the fecal coliforms, also respiratory tract *Pasteurellaceae* isolates (Catry et al., 2005) from calves originating from a high-density veal calf herds showed a higher degree of resistance than isolates from calves that originated from dairy herd or beef cattle herds. In-feed medication and milk replacers that are extensively used in veal calf herds, might be involved in the higher resistance levels, exerting a selection pressure through systemic distribution or through direct contact (nasopharynx or tonsils) with the microbiota of the upper respiratory tract (Catry et al., 2005).

Various authors have suggested a dilution effect by susceptible bacteria due to a soiled environment, resulting in a more diverse intestinal microbiota and thus less resistant strains. A greater proportion of *E. coli* isolated from pigs on pasture were sensitive to 13 antimicrobial agents tested than isolates from pigs housed in a farrowing house or concrete-floored finishing unit (Langlois et al., 1988). This phenomenon may be referred to as ‘environmental dilution’ and results in a microbial population with an equilibrium between susceptible and resistant subpopulations or even a predominance in susceptible bacterial populations. Hygienic measures play an ambiguous role in the

prevalence of resistance. In disease control, hygiene and sanitation are very important in preventing disease introduction and spread in a herd or flock and hence prevents an antimicrobial intervention (Cogliani et al., 2011). Furthermore, hygienic measures seem to help in avoiding the spread of resistance, for example between farms or within a farm (Laanen et al., 2013). On the other hand, hygienic standards could result in an exposure to a more resistant bacterial population . This was seen in broiler chickens and fattening pig farms where a lower hygiene standard in farms was associated with lower resistance in intestinal Enterobacteriaceae (Dewulf et al., 2007; Persoons et al., 2011).

Diet

The possible impact of diet on the prevalence of resistant intestinal bacteria has been suggested in several studies (Table 2). A change in the composition of diets may influence the intestinal environment, such as pH, and may thus act as a stressor for the intestinal microbiota (Alexander et al., 2008). This was demonstrated in cattle fed antimicrobial agents with silage- or grain-based diets. Animals on a grain diet with either tetracycline, doxyxycline, monensin or tylosin showed a higher prevalence of tetracycline resistant *E. coli* in fecal samples, compared to cattle on a silage diet with one of these antimicrobials (Alexander et al., 2008). A pH decrease in the rumen, after feeding a grain diet, might trigger the expression of membrane-bound transporters in *E. coli*, a common mechanism of tetracycline resistance (Roberts, 1994). Khachatryan et al. assumed a multifactor selective system for streptomycin-sulfadiazine-tetracycline (SSuT) resistant *E. coli* strains from dairy cattle (Khachatryan et al., 2006). Animals receiving a dietary vitamin D supplement showed a nearly twofold increase in the prevalence of SSuT resistant strains compared to animals that did not receive any supplement. The authors performed *in vitro* experiments, showing that SSuT resistant

strains attained a higher density of cells at stationary phase than non-SSuT resistant strains in the presence of the vitamin D additive. They concluded that the relationship between the prevalence of SSuT resistant strains and the vitamin D additive may be related to genetic linkage of the SSuT determinants to other genes, so called 'beneficial genes' that confer selective advantage in the presence of the vitamin D additive.

Further investigations are needed in order to determine how changes in diet composition may impact the prevalence of antimicrobial resistant enteric bacteria and thus the spread of resistance into the environment.

Bacterial factors

Bacterial virulence

In certain bacterial strains there is substantial evidence for a common mechanism for virulence and resistance or between particular virulence and resistance genes (Table 3). Besides common mechanisms of resistance and virulence, mobile genetic elements such as plasmids and integrative conjugative elements (ICE's) may carry both virulence and resistance genes, which can be concurrently transmitted between and within bacterial species (Beaber et al., 2004). For instance, avian pathogenic *E. coli* strains were shown to carry a conjugative R plasmid containing both resistance genes and virulence genes (Johnson et al., 2002). The hazard of these common mechanisms or linked occurrence of virulence and resistance genes can be defined as the possibility of co-selection of virulence genes by use of antimicrobials and consequentially maintenance of resistance in populations of pathogenic bacteria (Boerlin et al., 2005a). Yet, the mechanisms involved determine whether positive or negative associations between resistance and virulence take place (Beceiro et al., 2013; Martinez and Baquero, 2002).

Focusing on *E. coli,* there are numerous controversies on the link between virulence and resistance (Da Silva and Mendonca, 2012). For porcine enterotoxigenic *E. coli* (ETEC), diverse resistance and virulence genes profiles have been seen (Boerlin and Reid-Smith, 2008), resulting in varied outcomes on clustering of resistance and virulence genes. Furthermore, only few or no associations between resistance and virulence genes were reported in porcine multidrug resistant ETEC by Smith et al. (Smith et al., 2010). On the other hand, field data showed statistical associations between virulence and resistance genes for ETEC isolated from pigs (Boerlin et al., 2005b). This has been supported by the clustered prevalence of the tetracycline resistance gene *tetA* and several virulence factors on a common plasmid in porcine ETEC (Goswami et al., 2008). The latter confirmed the hypothesis that antimicrobial resistance is more common in ETEC than in other porcine *E. coli* (Boerlin et al., 2005b). Yet, where positive associations were found for *tetA*, this was not the case for certain virulence factors and *tetB*. Other studies report the presence of a pTC plasmid, linking resistance and enterotoxin virulence genes in porcine ETEC (Fekete et al., 2012), in F18-positive strains (Olasz et al., 2005) and in an *E. coli* O149:H10 strain shown to have enhanced virulence (Goswami et al., 2008). Studies on enterohemorrhagic *E. coli* (EHEC) in cattle (Valat et al., 2012) also aiming at investigating the possible link between resistance and virulence genes reported only few or no associations.

The AcrAB-TolC efflux pump, distributed in several Gram-negative species, expels antimicrobial agents, but also host-derived compounds with bactericidal activity such as fatty acids and bile salts (Perez et al., 2012). This efflux pump is also required for bacteria to be pathogenic (Kilroy et al., 2016) and to show resistance towards several classes, such as β-lactams, aminoglycosides, fluoroquinolones (Xavier et al., 2016), tetracyclines and macrolides (Martinez et al., 2009). For *Klebsiella pneumoniae* it has

been shown that porin deficiency can increase antimicrobial resistance, but decrease virulence at the same time (Tsai et al., 2011). Besides the recently-described plasmid-mediated colistin resistance (Liu et al., 2016; Hasman et al., 2015; Torpdahl et al., 2017), resistance to colistin in Gram-negative bacteria is caused by either the loss or by changes in their lipopolysaccharide (LPS), thereby preventing or reducing the affinity of polymyxins (Beceiro et al., 2014; Landman et al., 2008; Latorre et al., 2016; Xavier et al., 2016). A loss or change in LPS has been associated with a noticeable cost in terms of overall fitness and virulence in colistin resistant *Acinetobacter baumannii* (Beceiro et al., 2014; Lopez-Rojas et al., 2011). Relation with fitness will be discussed more in-depth in the respective section.

Field studies have shown more phenotypic resistance in bacteria from diseased animals than in bacteria from healthier animals, such as *in E. coli* from dairy calves with diarrhea when compared to healthy dairy calves (de Verdier et al., 2012), in *E. coli* from dairy cows with clinical mastitis (when compared with dairy cows with subclinical mastitis) (Suojala et al., 2011) and in *Streptococcus suis* from pigs with diverse clinical conditions (Li et al., 2012). Yet, findings on the statistical relationship between presence of virulence factors and resistance phenotypes in the field should be interpreted with caution. Though phenotypic resistance to one or more antimicrobials was associated with the presence of virulence genes in *E. coli*, none of these virulence factors were associated with the respective disease (de Verdier et al., 2012; Suojala et al., 2011), suggesting that detection of virulence factors might not always predict clinical outcome in field conditions (Li et al., 2012) and that other factors may explain higher resistance prevalences in diseased animals. For instance, higher prevalence of resistance in pathogenic isolates from diseased animals as a consequence of antimicrobial treatment has been suggested (Harada and Asai, 2010).

Recently, another connection between virulence and resistance to antimicrobials has been described in *Salmonella* Typhimurium where the expression of a type III secretion system, encoded by genes on the *Salmonella* pathogenicity island (SPI) 1, triggers gut tissue invasion followed by intracellular growth retardation and antimicrobial tolerance (Arnoldini et al., 2014; Diard et al., 2014). This results in the so called 'persister cells' with increased tolerance for antimicrobial agents and thus promoting persistence in an antimicrobial environment (Arnoldini et al., 2014; Diard et al., 2014). Upon cessation of antimicrobial treatment, former persister cells reseed the gut lumen and thereby facilitate disease transmissibility to new hosts (Diard et al., 2014).

Bacterial fitness

Another bacterial factor with an effect on AR spread is the fitness cost after having acquired a resistance determinant (Table 3), either by mutation (Giraud et al., 2003; Wichelhaus et al., 2002) or horizontal gene transfer (Johnsen et al., 2002). A loss in fitness can be reflected in a reduced growth rate (Andersson, 2006; Majcherczyk et al., 2008), a reduced transmission rate (Randall et al., 2008), a higher clearance rate (Gustafsson et al., 2003) and a decreased invasiveness (Fernebro et al., 2008). For instance, resistance to fluoroquinolones in *P. aeruginosa* can cause impaired motility (Stickland et al., 2010), and resistance to aminoglycosides can alter the structure of the ribosome (Holberger and Hayes, 2009; Springer et al., 2001). This could make the resistant strains less competitive than the susceptible ones and should result in a gradual reduction of resistance prevalence if no selection pressure is present. However, it is often observed that a reduction or discontinuation of antimicrobial use in farm environments does not necessarily result in a decreased prevalence of antimicrobial resistant isolates, at least not in the short term (Bunner et al., 2007; Enne et al., 2001; Khachatryan et al., 2004; Thakur and Gebreyes, 2005). To assess the

different antimicrobial mechanisms, different antimicrobial resistance elements were either introduced (through a plasmid or a transposon) or induced (through a chromosomal mutation) *in vitro* to a porcine *E. coli* isolate (Enne et al., 2005). *In vitro,* there was a fitness cost for the mutant strain and for the strain carrying the plasmid. When tested *in vivo*, the fitness cost imposed by the carriage of each antimicrobial resistance element studied was generally low or non-existent (Enne et al., 2005). To explain this *in vivo* difference, host factors and/or the competing microflora could be also involved in determining how well a particular *E. coli* strain is able to colonize the pig gut (Enne et al., 2005).

Some resistance-conferring mutations may even enhance the bacterial fitness (Luo et al., 2005). Enhanced fitness has been seen in a fluoroquinolone-resistant *Campylobacter jejuni* strain, directly linked to a single point mutation in *gyrA* that conferred high-level resistance to fluoroquinolones (Luo et al., 2005). Moreover, the carriage of a transposon (Tn1) conferring antimicrobial resistance improved fitness *in vivo* (Enne et al., 2005). Most likely, the insertion of Tn1 disrupted a gene that imposed fitness cost or the transposon itself conferring a fitness advantage. Apart from that, a mechanism of compensatory mutations has been described (Andersson and Levin, 1999) enabling a resistant strain to compensate for fitness loss and successfully compete with, or even prevail over susceptible strains (Handel et al., 2006; Johnsen et al., 2009). The level to which compensation is attained and the number of compensatory mutations depends on the bacterial strain, the resistance mechanism and the environmental conditions (Andersson and Hughes, 2010; Beceiro et al., 2013).

Antimicrobial resistance: a complex problem

H. L. Mencken once stated that "for every complex problem there is an answer that is clear, simple, and wrong" (Mencken, 1917). The complex nature of antimicrobial

resistance does not allow for over-simplification when dealing with this phenomenon, let alone when combatting it. Although the evolution of antimicrobial resistance was intensified mainly by antimicrobial use, numerous other factors have been shown to play an important role. The most worrisome fact is that all factors seem to be interrelated, jointly contributing to the emergence, selection and spread of antimicrobial resistance and rendering antimicrobial therapy less effective. Therefore, continued research is needed to further unravel on the underlying mechanisms through which these factors are connected.

Table 1. Chemical factors that contribute to the selection and spread of antimicrobial resistance (AR)

Factors	Reference	Study set up	Bacteria	Study focus	Resistance Mechanism	Main result
Biocides						
	McMurry & Levy, 1998	*In vitro*	*Escherichia coli (E. coli)*	Triclosan (TLN)	Lipid synthesis blockage by mutations or overexpression of the *fab*I gene	Intrinsically-resistant organisms to TLN may contain TLN-insentive enoyl reductases
	Levy, 2002	Review	*E. coli, Salmonella enterica (S. enterica)*	Quaternary ammonium compounds (QAC), TLN, Chlorexidine (CHX)	Efflux pumps, *mar* operon*, soxR, acrAB*	Role of MDR efflux pumps in biocide resistance
	Braoudaki & Hilton, 2004	*In vitro*	*E. coli O157, S. enterica*	benzalkonium chloride (BKC), TLN, CHX	Adaptive resistance	Special concern for inappropriate TLN use
	Karatzas et al., 2007	*In vitro*	*S. enterica* (serovar Typhimurium)	QAC, oxidizing compound blend (OXC), phenolic tar acids-based (TOP), TLN	*acrAB* efflux pump + reduced invasiveness	Exposure to QAC, TLN selects for *S.* Typhimurium
	Fraise, 2002	*In vitro*	*Staphylococcus aureus (S. aureus),* enterococci	CHX, QAC, Phenolic disinfectant (Stericol), TLN, Glutaraldehyde 2%,	-	Increased MIC, MBC indicate but not clearly show a correlation among biocide use and AR
	Chuanchuen et al., 2007	*In vitro*	*S. enterica*	BKC	*intI1* and *qacEΔ1* association	*QacE, qacEΔ1* genes contribute to BKC resistance, class1 integrons (*intI1*)
	Sidhu et al., 2001	*In vitro*	*Staphylococcus* spp.	QAC (BKC)	Plasmid mediated efflux pumps (*QacA/B, qacC/smr*) + *blaZ* on transposons	Presence of QAC and β-lactam antibiotics could lead to co-selection of AR
	Sidhu et al., 2002	*In vitro*	*S. aureus, cns-*staphylococci	QAC (BKC)	Plasmid mediated efflux pumps (*QacA/B*) + *blaZ* on transposons	Higher frequency of AR among BKC-resistant strains
	Slifierz et al., 2015a	*In vivo*	MRSA (swine)	QAC	*Qac* genes prevalence, the most common genotype was *qacG qacH smr*	All MRSA carried at least one *qac* gene
	Cocchi et al., 2007	*In vitro*	*E. coli*	*qacEΔ1 involved study (but did not test with QAC* substances*)*	Integrons (3 classes) mainly class 1	*E. coli* commensal strains are a source of AR determinants, 37% contain class1 integrons
	Russell & Houlihan, 2004	Review	*Pseudomonas aeruginosa (P. aeruginosa)*	TLN, QAC, CHX	-	No link has yet been seen between continuous use of biocides in communities and higher AR
	Beaber et al., 2004	*In vitro*	*E. coli, Vibrio cholera (V. cholera)*	DNA damage, regulate conjugative transfer of AR genes in bacterial population	Integrating conjugative elements (ICE). SOS response to DNA damage for horizontal dissemination of AR genes	*SetR* a repressor gene from SXT (an ICE from *V. cholera*) is alleviated from SOS response. ciprofloxacin and trimethoprim induces trimethoprim/sulphamethoxazole transfer.

	Gillings, 2010	Review	*Betaproteobacteria*	QAC	Evolutionary history of Class 1 integrons, role of transposons (capturing Integrons with a *qac* cassette)	Indiscriminate use of biocides might result in the selection of genetic elements that increase AR
Heavy metals						
	Cavaco et al., 2011	*In vitro (dust samples)*	Methicillin-resistant *S. aureus (*MRSA)	Zinc chloride, Copper sulphate	*czrC* resistance gene	Almost all zinc-resistant MRSA carried *czrC* gene. Use of zinc in feed might contribute to the emergence of MRSA. MICs of $CuSO_4$ not associated with methicillin resistance or *czrC* gene. No MSSA contained *czrC* gene
	Argudin et al., 2016	*In vitro*	Livestock associated (LA) MRSA	Arsenic compounds, cadmium, copper, zinc, staphylococcal cassette chromosome (*SCC*)mec	Metal resistance genes (*arsA,cadD, copB, czrC*) among LA *S. aureus* isolates	Most LA-MRSA isolates were positive for one at least metal-resistance gene. *czrC* gene was found in the presence of *SCC mec V.*
	Li et al., 2011	*In vitro*	Clonal complex (CC) 398 MRSA	Novel types of *SCCmec*	Types V, IX and X of *SCCmec*	Types V (subtype c), IX and X of *SCCmec* carried genes conferring metal resistance
	Amachawadi et al., 2015	*In vivo*	*mecA*-positive MRSA (swine)	In pigs, dose-response to zinc supplementation in pigs	*mecA* and *czrC* genes prevalence	Presence of *mecA* and *czrC* genes were positively associated with higher levels of Zn
	Moodley et al., 2011	*In vivo*	MRSA ST398 (swine)	In pigs, dose-response to zinc supplementation in pigs	Quantification of MRSA ST398	Feed supplemented with tetracycline or zinc increased MRSA ST398 population
	Slifierz et al., 2015a	*In vivo*	MRSA (swine)	Zinc oxide supplementation of pig feed	*czrC* gene prevalence and persistance	High doses of zinc oxide can increase prevalence and persistence of MRSA
	Slifierz et al., 2015b	*In vivo*	MRSA (swine)	Use of heavy metals in pigs	*czrC* gene prevalence	In-feed concentrations of zinc and frequent disinfection of nursery pens are associated with MRSA shedding
	Delmar et al., 2014	Review	*E. coli*	Copper, Silver resistance	Bacterial multi-drug efflux transporters (CusCFBA complex the only RND transporter), enzymatic alteration	Pump components of Cus system (efflux machinery. Enzymes that modify copper and silver (e.g. Cu(I) to less toxic Cu(II))
	Hasman and Aarestrup, 2005	*In vitro*	*Enterococcus faecium (E. faecium)*	Copper suphate, glycopeptides, macrolide	*tcrB* gene (copper resistance), *Tn1546* (glycopeptide res.), *ermB* (macrolide res.)	*tcrB* gene was located closely upstream of the *Tn1546* element.
	Mata et al., 2000	*In vitro*	*Listeria monocytogenes (L. monocytogenes)*	Macrolides, cefotaxime, $ZnSO_4$, $CoCl_2$, K_2CrO_4, $CdSO_4$, $CuCl_2$, $NaAsO_2$	*mdrL* gene encoding a multidrug efflux transport system	When disrupted, lower MIC's for $ZnSO_4$, $CoCl_2$, K_2CrO_4.
	Cavaco et al., 2010	*In vitro*	MRSA CC398	Zinc, Cadmium, Sodium arsenate, Copper sulphate, Silver nitrate resistance	*czrC gene, l*ocated within the clonal complex SCCmec type V	Zinc, Cadmium resistance may play a role in co-selection of methicillin resistance in *S. aureus*
	Medardus et al., 2014	*In vivo*	*Salmonella* spp	Copper, Zinc resistance	*pcoA* (copper), *czcD* (zinc) genes	Strong association between heavy metal tolerance and AR among *Salmonella* spp.

Table 2. Factors related to animal husbandry that contribute to the selection and spread of antimicrobial resistance (AR)

Factors	Reference	Study set up	Bacteria	Study focus	Study results	Other remark(-s)
Animal factors						
	Sorum and Sunde, 2001	Review	Normal flora bacteria, focus on *E. coli*	Use of AMs, stress from temperature, crowding, management	Heat stress, crowding, and management also contribute to the occurrence of AR	Normal flora can be a reservoir of AR genes. The goal is to use normal flora to actively protect against infectious diseases
	Verbrugghe et al., 2012	Review	*P. aeruginosa, E. coli, S. enterica, Campylobacter jejuni,* Mycobacteria	Chronic stress	Increased availability of iron (through the intervention of neuroendocrine (stress) hormones). Catecholamines influence *E. coli, P. aeruginosa, Salmonella* spp. by quorum sensing mechanisms. Roles of host immune system and intestinal barrier	Stress may influence the outcome of an infection. Stress promotes the colonization of farm animals by enteric pathogens such as *E. coli, S. enterica* and *Campylobacter.*
	Moro et al., 2000	*In vivo*	*E. coli* (swine)	Heat stress	4 studies, 1: heat stresses increased levels of resistance, 2: *E. coli* isolates with higher AR prevalence reside in ileum and caecum compared to colon and rectum. 3+4: increase in intestinal motility and peristalsis produce an outflow of resistant *E. coli* organisms from the upper to lower intestinal tract.	Heat stress increases ampicillin and tetracycline resistance.
	Mathew et al., 2003	*In vivo*	*E. coli* (swine)	Apramycin treatment. Study of effects of various stressors: management, cold stress, heat stress, overcrowding, intermingling, poor sanitation, intervention with oxytetracycline	MICs of isolates from control pigs receiving apramycin returned to pretreatment levels following removal of the antibiotic, whereas isolates from pigs in cold stress, overcrowding, and oxytetracycline groups expressed greater MIC's	Factors promoting AR: cold, heat, crowd, sanitation, oxytetracycline administration, apramycin exposure(effect only during treatment), intermingled animals
	Berge et al., 2005	*In vivo*	*E. coli* (cattle)	Single dose florfenicol and AR patterns and effect of weaning	Shift towards carriage of more multi-resistant *E. coli* after florfenicol treatment. Animals weaned one month prior transportation had lower levels of multi-resistant strains compared to those that were transported immediately after weaning	Long after treatment, the resistance to chloramphenicol persisted. Level of resistance was influenced by complex interaction of animal source and previous management
	Khachatryan et al., 2004	*In vivo*	*E. coli* (dairy calves)	Age of animals (+administration of low dose of oxytetracycline)	Active selection for traits linked to SSuT phenotype led to persisting AR *E. coli* populations in dairy calves	Shedding of SSuT resistant strains was greater than susceptible strains in neonatal claves. No difference in older animals
	Dewulf et al., 2007	*In vivo* (field study) and questionnaire	Lactose-positive enteric coliforms (swine)	Housing, management and antimicrobial consumption	Besides antimicrobial use (AU), factors like inside pen hygiene can influence the development and maintenance of AR bacteria.	Tetracycline-resistance in *E. coli* is linked to resistance against ampicillin and trimethoprim-sulphonamides

	Langlois et al., 1988	*In vivo*	Coliforms (swine)	Effect of age and housing location on AR	The proportion of resistant isolates was higher in pigs 6 months of age or less and lower in pigs on pasture.	Exposure to antibiotics is not the only factor influencing the prevalence of AR
	Akwar et al., 2008	*In vivo*	*E. coli* (swine)	Different in-feed medication policies on AR	AR more frequent in farms using in-feed medication, and more frequent in weaner pigs compared to finisher pigs.	Fecal *E. coli,* a potential reservoir of AR genes. Use of medication in swine provides selective pressure for AR in *E. coli* in pigs
	Van den Bogaard et al., 2002	*In vivo*	Enterococci, focus on vancomycin-resistant enterococci (VRE)	AR prevalence patterns from: a) chicken populations: broilers and laying hens. b) human populations: broiler farmers, laying-hens farmers, poultry slaughterers	AR prevalence higher in broilers and in broiler farmers. Resistance in broilers correlated with broiler farmers and poultry slaughterers. In all VRE isolates, *vanA* gene was isolated.	Transfer of AR through transposons seems to occur more frequently. For VRE transmission of AR might be through clonal transmission of animal strains.
	Walk et al., 2007	*In vivo*	*E. coli* (dairy cattle)	Farm type (conventional vs organic), age of cattle (cow vs calf), bacterial phenotype (A, B1, B2, D)	Organic farming practices not only change the frequency of resistant strains but also impact the overall genetic composition of the *E. coli* flora. Prevalence of *Tet*^{r} loci on dairy farms has little to do with the use of tetracycline.	Evidence for clonal resistance (ampicillin resistance), genetic hitch-hiking (*Tet*^{r}). Association between low multidrug resistance, organic farms and strains from phylogenetic group B1.
Farm factors						
	Berge et al., 2005	*In vivo*	*E. coli* (calves)	Effects of calf age, farm-type and individual-calf treatment	Resistant *E. coli* more likely from : a) calves 2 weeks old more than day-old, 4-week-old and 6-week-old calves, b) treated with antibiotics within 5 days prior to sampling vs non-treated animals	Dynamics of multiple antibiotic-resistance patterns in large bacterial populations studied with combination of cluster analysis with multinomial statistical methods
	Berge et al., 2010	*In vivo*	*E. coli* (cattle)	Geographic, farm and animal factors associated with multiple AR (MAR)	MAR in fecal *E. coli* isolates from cattle was influenced by factors not directly associated with the use of antibiotics, including geographic region, animal age and purpose (beef vs dairy)	A generalized estimating equations cumulative logistic regression model was used to identify factors associated with an increase in MAR
	Catry et al., 2005	*In vivo*	*Mannheimia haemolytica (M. haemolytica), Pasteurella multocida (P. multocida)* (calves)	Different herd types and acquired AR.	Different herd types and association with acquired AR	Calves for fattening show a higher prevalence of AR than in isolates from dairy or beef calves
	Langlois et al., 1988	*In vivo*	Coliforms (swine)	Effect of age and housing location on AR	The proportion of resistant isolates was higher in pigs 6 months of age or less and lower in pigs on pasture	Exposure to antibiotics is not the only factor influencing the prevalence of AR
	Dewulf et al., 2007	*In vivo* and questionnaire	Lactose-positive enteric coliforms (swine)	Housing, management and antimicrobial consumption	Besides AU, factors like pen hygiene can influence the development and maintenance of AR bacteria.	Tetracycline-resistance in commensal *E. coli* is often linked with resistance to other antimicrobial drugs like ampicillin and trimethoprim–sulphonamides.

	Persoons et al., 2011	*In vivo* and questionnaire	*E. coli* (broilers)	Farm level and animal level factors for ceftiofur resistance	Management factors influencing ceftiofur resistance occurrence	Animal level: Resistance to amoxicillin and to trimethoprim-sulphonamide influences for ceftiofur resistance
Diet						
	Alexander et al., 2008	*In vivo*	*E. coli* (cattle)	Effects of sub-therapeutic administration of chlorotetracycline+sulfamethazine, chlorotetracycline, virginiamycin, monensin, tylosin or control.	Irrespective of treatment, the prevalence of cattle shedding TET-res *E. coli* was higher in animals fed grain-based compared to silage-based diets.	Sub-therapeutic administration of tetracycline in combination with sulfamethazine increased the prevalence of tetracycline- and AMP-resistant E. coli in cattle.
	Khachatryan et al., 2006	*In vivo* and *in vitro*	*E. coli* (calves)	Dietary supplement and tetracycline and streptomycin, sulphadiazine, tetracycline (SSuT) resistance	Dietary supplement and its vitamin D component supported higher cell density of SSuT strains.	No evidence for contribution of oxytetracycline for SSuT resistance

Table 3. Bacterial factors that contribute to the selection and spread of antimicrobial resistance (AR)

Factors	Reference	Study set up	Bacteria	Study focus	Resistance mechanisms	Main result(-s)
Virulence	Boerlin et al., 2005	*In vitro*	*E. coli* (swine), enterotoxigenic *E. coli* (ETEC) and non-ETEC isolates	Virulence genes for LT(*elt*), STa (*estA*), STb (*estB*), F4 (*faeG*), F5 (*fanA*), F6 (*fasA*), F18 (*fedA*), Stx2e (*stxe2*) and resistance genes (*tetA, tetB, sul1, sul2, sul3, aadA, strA, strB, aac(3)IV*)	Statistical associations between AR genes and virulence genes (e.g. OR 21.5 for *elt* and *tetA*, OR 28.68 *faeG* and *aac(3)IV*). *paa* and *sepA* predominant among ETEC isolates	Antimicrobial use (AU) may select for bacteria carrying virulence genes. AR genes may be stabilized and fixed in pathogen populations by their linkage to virulence genes
	Martinez & Baquero, 2002	Review		Genetic linkage between resistance and virulence	Virulence determinants encoded by AR plasmids (heat-stable enterotoxin, heat-labile enterotoxin, aerobactin, serum resistance, hemolysin, microsin D93, colicins, bacteriocin, *V. cholera* cytotoxin, cell invasiveness, *vir* plasmids pZM3, Dt66, pTE195) or by phages (Shiga toxin, adhesion factor, pathogenicity island and type IV pilus, cholera toxin, exotoxin A (*Strept. pyogenes))*	Strategies against virulence may reduce AR. Yet, microbial evolution is not a reversible process
	Beceiro et al., 2013	Review		AR and fitness and their effect on virulence.	Plasmids, phage-mediated transduction, outer membrane vesicles, cell wall modifications, efflux pumps, two component regulatory systems	Association between AR and virulence. Novel anti-virulence therapies (cell-to-cell signalling inhibitors, RND efflux pump inhibitors, antimicrobial compounds with anti-virulence activity)
	Da Silva and Mendonca, 2012	Review	*E. coli*	Association between AR and virulence in *E. coli*	DNA mutation, horizontal gene transfer (HGT)	A link between resistance and virulence seem to exist (*e.g.* as seen in *E. coli* ST131 clone) yet there are controversies among studies
	Perez et al., 2012	*In vitro*	*Enterobacter cloacae*	*AcrAB-TolC* efflux pump in resistance, fitness and virulence	Strains lacking *acrA* and *tolC* gene were associated with higher decrease in antibiotic MIC's than strains lacking only *tolC* gene). *acrA* and *tolC* genes are key to the fitness of *E. cloacae*	*AcrAB-TolC* components play a key role in AR, biological competitiveness and are required for full virulence of *E. cloacae*
	Kilroy *et al.*, 2016	*In vivo*	*Salmonella enteritidis* (avian)	*AcrAB, acrEF, mdtABC, tolC* role in virulence	Vaccination of targeted gene deletion (Δ*tolC*, Δ*acrABacrEFmdtABC*) mutant strains	The mutant strains significantly protected against gut and internal organ colonization
	Martinez et al., 2009	Review		Efflux pumps (RND, MATE, MFS, SMR, ABC)	Genes coding for MDR efflux pumps are present in bacterialchromosomes, are highly conserved andtheir expression are tightly regulated	Efflux pump roles: detoxification of intracellular metabolites, bacterial virulence, cell homeostasis and intracellular signal trafficking
	Tsai et al., 2011	*in vitro* and *in vivo*	*Klebsiella pneumoniae* (mice)	Outer membrane porins *OmpK35* and *OmpK36* AR and virulence	Single deletion of *ompK36* resulted in MIC shifts of cefazolin, cephalothin and cefoxitin from susceptible to resistant. Deletion of *ompK35* had no significant effect. Double deletion further increased the MICs	Porin deficiency in *K. pneumonia* could increase AR but decrease virulence at the same time

Landman et al., 2008	Review	*Acinetobacter baumannii, P. aeruginosa* and Enterobacteriaceae	Polymixins	Resistance to colistin in Gram-negative bacteria is caused either by loss or by changes in their lipopolysaccharide (LPS), preventing or reducing the affinity of polymyxins	Loss or changes in bacterial LPS can lead to reduced fitness and virulence.
Beceiro et al., 2014	*In vitro* and *in vivo*	*A. baumannii* (mice)	Colistin	2 mechanisms: loss of LPS (Δ*lpx* mutants) and phosphoethanolamine addition to LPS mediated through mutations in *pmrAB*. Both showed a reduction in fitness	Bacterial fitness and virulence are lower in the presence of colistin treatment.
Lopez-Rojas et al., 2011	*In vitro* and *in vivo*	*A. baumannii* (mice)	Colistin	Resistance due to decreased binding to the bacterial outer membrane because of LPS remodelling caused by changes in PhoPQ and PmrAB	Resistance to colistin in *A. baumannii* is associated with lower *in vivo* bacterial fitness and decreased virulence.
Smith et al., 2010	*In vivo*	*MDR E. coli* (swine)	AR and virulence gene profiles in MDR ETEC from pigs with post-weaning diarrhoea	The most prevalent serotypes were O149, O141 and Obt. O141 showed elevated prevalence of aminoglycoside resistance genes and possessed more virulence genes	Various selection pressures at the individual farm level rather than emergence and lateral spread of MDR resistant/virulent clones
Goswami et al., 2008	*In vivo*	*E. coli O149:H19* (swine)	Effect of plasmid *pTENT2* on severity of porcine post-weaning diarrhoea by an O149 ETEC	*tetA* and virulence genes (*estA, paa, sepA*) present in pTENT2 plasmid. 2 groups (containing or not the plasmid) and clinical and pathological changes were compared	Animals with pTENT2-positive *E. coli* trains showed more severe symptoms. No difference in the persistence of ETEC between groups
Fekete et al., 2012	*In vitro*	Enterotoxigenic *E. coli* (porcine strain)	Plasmid *pTC* (encodes the *STa* and *STb* heat-stable enterotoxins and tetracycline resistance) sequencing	Five regions: i. maintenance region, ii. Toxin specific locus (*estA* and *estB* genes), *Tn10* transposon encoding tetracycline resistance, iv. *Tra* transfer region and v. coE1-like origin of replication	*pTC* is a plasmid containing an antimicrobial resistance locus, thereby representing a selection advantage for spread of pathogenicity in the presence of antimicrobials; increased disease potential
Olasz et al., 2005	*In vivo*	Enterotoxigenic *E. coli* (porcine strain)	Plasmid *pTC* (encodes the *STa* and *STb* heat-stable enterotoxins and tetracycline resistance)	Characterization of *pTC* and determination of its origin of replication	*pTC*-like plasmids are widely distributed among porcine ETEC strains. Co-evolution of AR and virulence in pathogenic *E. coli*
Valat et al., 2012	*In vitro*	Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* (cattle)	ESBL genes, phylogenetic grouping and virulence factor.	ESBLs mostly CTX-M1 and CTX-M-9. ESBL isolates mainly belonged to phylogroup A and lesser to D and B1. VFs higher in phylogroup B1 than A and D. almost all VFs found in CTX-M-1, lesser in CTX-M-9 and CTX-M-2	B1 was the most virulent phylogroup but not the most prevalent group, suggesting host-specific distribution of virulence determinants among phylogenetic groups
Johnson et al., 2002	*In vitro*	*E. coli* (avian)	Increased serum survival gene and selected virulence traits on a conjugative R plasmid.	This plasmid contains sequences with homology to *tsh* (associated with virulence), *intI1 (*encoding integrase of Class 1 integrons)	When transferred to an avirulent strain by conjugation enhanced its AR but not its virulence.
De Verdier et al., 2012	*In vivo* + question-naire	*E. coli* (calves)	Phylogeny, AR and virulence factors	Virulence genes *espP, irp* and *fyuA* more common in resistant *E. coli* than susceptible isolates. *terZ* virulence gene was associated with calf diarrhoea	More factors than AU influence the epidemiology of resistant *E. coli.*
Suojala et al., 2011	*In vivo* + question-naire	*E. coli*	Phylogeny, AR and virulence factors isolated in bovine mastitis associated to clinical signs	Majority of strains belonged to phylogeny group A which consisted mainly of commensal strains. Most common virulence genes *irp2, iucD, papC iss*	None of the studied phylogeny groups, virulence factors or AR traits were associated with clinical signs, persistence or clinical recovery from mastitis

	Li et al., 2012	*In vitro*	*Steptococcus suis* (swine)	AR, serotypes and virulence factors (VFs)	Most prevalent VFs: *muramidase-released protein, suilysin* and *extracellular factor.* Serotype 2 the most prevalent. MDR (>2 drugs) present in 98.73% of the isolates	Presence of VFs was associated with certain AMR phenotypes.
	Harada & Asai, 2010	Review	*E. coli*	Role of AU and other factors (co-resistance, cross-resistance, virulence factors, serotypes) on AR prevalence	Host animals and bacterial properties (virulence, serotypes) affect the occurrence and prevalence of AR *E. coli* under the selective pressure from antimicrobial usage	Relationship between AU and AR over time. Also underline other factors (cross-resistance and co-resistance effects)
	Arnoldini et al., 2014	*In vitro*	*Salmonella* Typhimurium	Bi-stable expression of virulence genes, leads to phenotypically virulent and avirulent subpopulations	Expression of virulence factors often entail metabolic costs and the resulting growth retardation could generally increase tolerance against antibiotics	Virulent population increased survival after exposure to antibiotics.
	Diard et al., 2014	*In vitro* and *in vivo*	*Salmonella* Typhimurium (mice)	Antibiotic treatment and virulence	*In vivo,* while avirulent mutants take over the gut lumen and abolish disease transmission in untreated mice, ciprofloxacin tilts the balance in favor of virulent, wild-type bacteria	Antimicrobial treatment can promote cooperative virulence during within-host evolution, increase duration of transmissibility, and thereby enhance the spread of an infectious disease.
Fitness	Wichelhaus et al, 2002	*In vitro* and *in vivo*	*Staphylococcus aureus (S. aureus)*	Biological cost of rifampin resistance	*In vitro* selected strains showed a low reduction in fitness. Seven different *rpoB* genotypes	Most prevalent mutation was 481His→Asn was not associated with fitness cost
	Giraud et al, 2003	*In vitro* and *in vivo*	*Salmonella enterica*	Cost on fluoroquinolone resistance	Resistance acquired through mutation. Partial reversal of fitness cost observed, which was not associated with the loss of *gyrA* mutations.	Mutants had growth defects on agar, normal generation times in liquid culture and successfully colonization of the gut
	Johnsen et al., 2002	*In vitro* and *in vivo*	Glycopeptide-resistant enterococci (GRE)	Plasmid-encoded *VanA* glycopeptide resistance	GRE with *VanA* resistance had a 4% reduction in fitness	Environmental adaptation, *in vivo* gene transfer and plasmid maintenance system exceeded the biological cost in all strains.
	Andersson, 2006	Opinion article	-	Biological cost of mutational antibiotic resistance	Compensatory evolution, cost-free resistance mechanisms (rare), genetic linkage or co-selection between resistance markers and other selected markers	Reduced growth rate
	Majcherczyk et al., 2008	*In vitro* and *in vivo*	Glycopeptide-intermediate *S.aureus* (GISA)	Glycopetide resistance and loss of infectivity	GISA are characterized by multiple changes in the cell wall, altered expression of global virulence regulators	In rats, GISA showed attenuated virulence and reduced bacterial fitness
	Gustafsson et al., 2003	*In vivo*	*E. coli,* enterococci spp., α-streptococci, coagulase-negative staphylococci	Prolonged antibiotic treatment and effect on AR and mutation frequency for rifampicin resistance and streptomycin resistance.	Different mutation frequency to rifampicin resistance	High AU selected for commensals with highly increased resistance and a slight increase in mutation frequency
	Fernebro et al., 2008	*In vitro*	*Streptococcus pneumoniae*	Influence of defects affecting *in vitro* growth rate	Growth defective mutants showed reduced invasiveness. Polarity effects on *yrdC* gene to mediate growth defect	Large fitness defects are needed to completely prevent pneumococci from causing invasive disease. Still, they cannot prevent colonization of the upper airways.

Khachatryan et al., 2004	*In vitro and in vivo*	*E. coli* (dairy calves)	SSuT *E. coli* isolates consistently out-competed susceptible strains *in vitro* and *in vivo*	Active selection for traits linked to SSuT phenotype led to persisting AR *E. coli* in dairy calves	Shedding of SSuT resistant strains was greater than susceptible strains in neonatal calves. No difference in older animals
Enne et al., 2001	*In vivo*	*E. coli* (humans)	Sulphonamide resistance levels after a national prescribing restriction campaign.	Lack of decline of sulphonamide resistance. Role of compensatory mutations, time, non-human use of sulphonamides. *sul*II genes more frequent than earlier	Genetic linkage of sulphonamide resistance to other resistance determinants
Thakur & Gebreyes, 2005	*In vivo*	*Campylobacter coli* (porcine)	AR prevalence in conventional and antimicrobial-free production systems	No reduction of AR prevalence in antimicrobial-free environment	High prevalence of AR in both conventional and antimicrobial-free production systems
Bunner et al., 2007	*In vivo*	*E. coli* (porcine)	AR prevalence in conventional and antimicrobial-free production systems	Cessation of AU did not result in immediate reduction in AR	Studies of long-term AU or cessation are needed to measure the rates of reversibility (if any) of AR.
Luo et al., 2005	*In vivo*	*C. jejuni* (chickens)	*In vivo* fitness, fluoroquinolone resistance	Enhanced fitness of fluoroquinolone-resistant strains in the absence of antibiotic selection pressure. The fitness advantage was due to the single point mutation in *gyrA*	When mono-inoculated into the host, resistant and susceptible strains showed similar levels of colonization and persistence. When co-inoculated, resistant strains outcompeted the susceptible strains
Andersson & Levin, 1999	Opinion paper	-	Biological cost of AR	Most resistance-determining mutations and accessory elements engender some fitness cost	However, fitness costs are likely to be ameliorated by subsequent evolution
Johnsen et al., 2009	Review + case study	-	Factors affecting the reversal of AR. Persistence of glycopeptide-resistant enterococci 12 year after avoparcin ban	Fitness cost reduces the frequency of resistance. Compensatory evolution decreases the fitness cost of resistance. Population processes counteract the reversal of resistance (persistence)	Resistance determinants may persist at low but detectable levels for many years in the absence of the corresponding drugs
Handel et al., 2006	Theoritical model	-	Compensatory mutations and resistance emergence on a population level	With compensatory mutations, resistance emergence is faster and more likely	
Beceiro et al., 2013	Review	-	Bacterial virulence and fitness affected by AR, co-selection, compensatory mechanisms and global responses	AR depends on the bacterial strains involved, virulence and resistance mechanisms, the ecological niche and the host	The association between virulence and resistance is becoming more beneficial for pathogenic bacteria
Andersson & Hughes, 2010	Review	-	Chromosomal mutations and fitness costs, plasmid mediated resistance genes and fitness costs, environmental conditions	Compensatory evolution role. It can occur by reversion of a mutation or by gene conversion. Also by gene amplification	Fitness cost can be reduced by regulation of the resistance mechanism. . Rate of reversibility of AR is slow
Cottell et al., 2012	*In vitro*	*E. coli*	Transferable ESBL resistance in the absence of antibiotic pressure	An absence of antibiotic pressure and inactivation of the antibiotic resistance gene had no effect on plasmid persistence, conjugation frequency, or bacterial-host biology	The persistence of AR genes and their vectors is to be expected in the absence of antibiotic selective pressure regardless of antibiotic use

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SCIENTIFIC AIMS

Chapter 2. Scientific Aims

Antimicrobial agents have been used for treating disease, preventing disease and improving feed efficiency in farm animals for more than six decades. During the last twenty years, the alarmingly increasing resistance prevalences to antimicrobial agents have urged both science and public health agencies to respond by acquiring knowledge on the origins and mechanisms of antimicrobial resistance and by monitoring antimicrobial use and antimicrobial resistance prevalence in pathogenic and commensal bacteria. However, there is still a lack of knowledge on the epidemiology of antimicrobial resistance and the factors contributing to the spread and selection of antimicrobial resistance at animal and population level.

Accordingly, the **overall aim** of this thesis was to gain insights in the epidemiology of antimicrobial resistance by conducting epidemiological studies.

Using data from various national monitoring reports, the **specific aims** of this thesis were:

a. to evaluate associations between veterinary antimicrobial use and antimicrobial resistance rates in food producing animals using publicly available data (Chapter 3)

b. to compare antimicrobial resistance prevalence among commensal *E. coli* strains and pathogenic *E. coli* strains from food producing animals in Belgium (Chapter 4)

Fluoroquinolones –a critically important antimicrobial class for human and veterinary medicine- have been widely used in poultry for more than two decades leading to high levels of fluoroquinolone resistance. The mechanisms governing fluoroquinolone resistance (*e.g.* step-wise chromosomal mutations, horizontally-transferred genes that

confer low levels of resistance) reveal the importance of acknowledging various factors (*e.g.* bacterial fitness costs, effects of different treatment schemes) when describing this phenomenon. Due to the worrisome increasing prevalence of fluoroquinolone resistance in poultry, the aim was to design standardized poultry models and test various factors for their *in vivo* effect in the selection and spread or fluoroquinolone resistance. Thus, the **specific aims** were:

c. to identify factors contributing to fluoroquinolone resistance selection in commensal *E. coli* strains in controlled *in vivo* poultry models (Chapter 5)

d. to evaluate the effect of a commercial competitive exclusion product on the selection and spread of fluoroquinolone resistance in commensal *E. coli* strains in controlled *in vivo* poultry models (Chapter 6)

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Chapter 3. Correlation Between Veterinary Antimicrobial Use And Antimicrobial Resistance In Food-Producing Animals: A Report On Seven Countries

Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries

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Abstract

Objectives: Based on data from publicly available national or international monitoring reports of seven European countries, correlations between antimicrobial use in food-producing animals and resistance prevalence for commensal *Escherichia coli* isolates originating from pigs, poultry and cattle, for eight antimicrobials were evaluated.

Methods: The link between the quantity of antimicrobials administered to food producing animals per country (expressed in mg/population correction unit) and the prevalence of resistance (interpreted by EUCAST epidemiological cut-off values) in *E. coli* isolates (4831 isolates in total) to antimicrobial agents representing the different antimicrobial classes used, was studied by means of polynomial regression analysis and the determination of the Spearman's rank correlation coefficient.

Results: A quadratic regression best fitted the antimicrobial use and antimicrobial resistance data. The coefficient of determination was, in decreasing order, 0.99 for fluoroquinolones and amphenicols, 0.94 for third-generation cephalosporins and sulphonamides, 0.93 for aminopenicillins, 0.81 for streptomycin and 0.80 for gentamicin and tetracycline. Spearman's rank correlation coefficient was 1 for amphenicols, 0.96 for sulfonamids, 0.93 for streptomycin and tetracycline, 0.89 for aminopenicillins, 0.86 for fluoroquinolones, 0.71 for gentamicin and 0.70 for third-generation cephalosporins.

Conclusions: These remarkably high coefficients indicate that, at a national level, the level of use of specific antimicrobials strongly correlates to the level of resistance towards these agents in commensal *E. coli* isolates in pigs, poultry and cattle. However, data restraints reveal the need for further detail in collection and harmonization of resistance and use data in Europe.

Introduction

Discussion on possible links between antimicrobial use in the production of food animals and emergence of antimicrobial resistance in animals and potentially also humans dates back more than four decades to the Swann Report (Swann, 1969). Since then, many papers have dealt with this alarming topic (Soulsby, 2007; Snary et al., 2004; Barton, 2000; Wegener, 2003; Aarestrup, 2012). Transfer of resistance between animals, and animals to humans has been studied extensively (Barza, 2002; Jensen et al, 2004; Hammerum and Heuer, 2009) and biological mechanisms of gene transfer between animal and human bacteria have been described (Wegener, 2003; Smet et al. 2011; Vignaroli et al, 2011). On the other hand, there are studies that question the link between antimicrobial consumption in animals and prevalence of resistant isolates in humans (Phillips et al., 2004; De Jong et al., 2009; Mather et al., 2011). The World Health Organization (WHO) lists antimicrobial resistance as a global concern and underlines the importance of trustworthy national surveillance systems (WHO, 2012).

In Europe, throughout the last two decades, several EU member states have made great progress towards monitoring antimicrobial resistance in food-producing animals (Bager, 2000; DANMAP, 1998; Moreno et al., 2000; Schwarz et al., 2007; MARAN, 2012, NORM-VET, 2011; Martel et al, 2000; SVARM, 2012). However, only in recent years, after clear calls for the urgent need for harmonization of resistance monitoring programs (Wegener, 2003; Silley et al., 2011; Silley et al., 2012; Marion, 2012; White et al., 2001), are national reports being published that use more or less uniform methodology on antimicrobial susceptibility testing, allowing for better comparison between countries. The scientific guidance of the European Food Safety Authority

(EFSA) has likely helped (EFSA, 2012). Another step forward was the implementation of epidemiological cut-off values, set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In this regard, the EFSA stated that from 2013 onwards, reports on the occurrence of antimicrobial resistance in the indicator *E. coli* should become mandatory and be included in each national surveillance program of an EU member state (EFSA, 2012).

Regarding the knowledge of veterinary antimicrobial use in Europe, huge improvement has been made through the activities of the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). In 2012, the second ESVAC report was published presenting the results on antimicrobial consumption in animals in nineteen EU / EEA (European Economic Area) member states plus Switzerland for the year 2010 (ESVAC, 2012). The report revealed important information on the sales of antimicrobial agents per country and the percentage of sales attributed to each antimicrobial class.

Turnidge and Christiansen (2005) stated, regarding antimicrobial use and resistance in human medicine, that obvious correlation is not at all obvious when considered carefully. Several studies measuring correlations between antimicrobial use and resistance in human medicine have been published (Monnet et al., 1998; Riedel et al., 2007; Lopez-Lozano et al., 2000; Bronzwaer et al., 2002; van de Sande et al., 2008; Albrich et al., 2004). These studies show a large variation in data selection and study design making study comparisons difficult. At European level, a human medicine study correlating mean antimicrobial use in European countries and resistance in *Streptococcus pneumoniae* showed a positive correlation between both (Riedel et al., 2007). In veterinary medicine, Asai *et al* (2005) published a study communicating that

the overall usage of veterinary antimicrobials appears to contribute to the appearance of antimicrobial resistance in *E. coli* isolates from apparently healthy animals in Japan. Given the increased availability and comparability of data on antimicrobial resistance and antimicrobial use in animals, the aim of this study was to evaluate whether, based on publicly available data, correlations between antimicrobial use and antimicrobial resistance in commensal *E. coli* from various food producing animals in Europe could be identified.

Materials and Methods

Study design

Data selection

National or international reports, describing European data on either antimicrobial use in animals and antimicrobial resistance in *E. coli* from apparently healthy food-producing animals were examined. Only reports that collected and analysed the data and presented the results in a comparable manner were used.

Antimicrobial use data

Data on antimicrobial use were obtained from the ESVAC 2012 antimicrobial use report. In this report the annual sales figures in twenty European countries are reported in absolute values as well as in relation to the animal population present in the country. In those countries, antimicrobials can be administered for treatment and/or prophylaxis of infection in animals but, since 2006, a total ban on the use of antimicrobials for growth promotion is implemented (Casewell et al., 2003; European Commicion, 2005). The magnitude of the animal population is quantified by means of the population correction unit (PCU). PCU is a technical unit of measurement based on the estimated

weight at treatment of livestock and of slaughtered animals for food-producing animals (poultry, pigs, cattle, small ruminants, rabbits) and horses in the corresponding year. The PCU also corrects for import and export of animals. One PCU is a representation of one kg of different categories of livestock and slaughtered animals (ESVAC, 2012; Grave et al., 2012). In our study, mg per PCU (mg/PCU), was used as a measurement of the antimicrobial use, for every veterinary antimicrobial class.

Antimicrobial resistance data

Concerning data on antimicrobial resistance, only countries that provide data on poultry, pigs and cattle from apparently healthy animals originating from studies conducted during 2010-2011 and for which at least ten isolates per animal species were available were included. For cattle, some countries only provided data on adult cattle (Austria, Norway), whereas Sweden and Switzerland only provided data on veal calves and Belgium, Denmark and the Netherlands provided data both on adult cattle and veal calves. Additionally, as instructed by EFSA and also proposed by several scientific reports (Silley et al., 2011; de Jong et al., 2012), only quantitative national results providing minimum inhibitory concentrations (MIC) were included. Antimicrobial susceptibility results were based on EUCAST epidemiological cut-off values (EUCAST, 2013), which determine whether a specific isolate is wild-type in relation to a particular antimicrobial and can be used to describe and quantify biological resistance, regardless of clinical efficacy (Cornaglia et al., 2004; Callens et al., 2012).

Based on the literature search and the selection criteria, data from seven European countries met the requirements to be included in the study. The data sources were a) EFSA and ECDC joint scientific report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2010 (EFSA, 2012), b) CODA-

CERVA report on zoonotic agents in Belgium: trends and sources 2010-2011 (CODA-CERVA, 2012), c) DANMAP 2011 report- Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark (DANMAP, 2011), d) MARAN 2011 and 2012 report- Monitoring of Antimicrobial Resistance and Antibiotic Use in Animals in the Netherlands (MARAN, 2012), e) NORM-VET 2011 report– Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (NORM-VET, 2011), f) SVARM 2011 report- Swedish Veterinary Antimicrobial Resistance Monitoring report (SVARM, 2012).

Data handling and data preparation

Based on the ESVAC 2012 report , the exact sales data for antimicrobials cannot be determined for each farm animal species separately. On the other hand, the antimicrobial resistance studies are conducted on certain animal categories and results are reported per animal category. To account for this discrepancy, we calculated an overall antimicrobial resistance prevalence for each of the antimicrobials, tested as the average prevalence obtained from the available data in the four major food-producing animal categories (poultry, pigs, cattle, veal calves). To calculate this average prevalence, we summed the number of resistant isolates and divided this by the sum of the total number of isolates tested from each of the four categories.

The ESVAC report on antimicrobial consumption provides data on the use in all farm animal species present in a country and calculates the PCU based on the population data of pigs, poultry, cattle, small ruminants, rabbits, fish and horses while there were just three or four animal categories (broiler chickens, pigs, cattle and veal calves) that composed the animal population of the resistance studies. However, among the countries included in our study, the sum of PCUs for pigs, adult cattle, veal calves and

poultry (broiler chicken) accounted for approximately 88% of the total PCUs in food producing animals (ESVAC, 2012; Eurostat, 2012). We therefore concluded that it was justified to compare both data sources, keeping this partial incongruence in mind.

The ESVAC report provides the antimicrobial use data per antimicrobial class (e.g. aminopenicillins) whereas the antimicrobial resistance data apply to specific molecules representing its corresponding antimicrobial class, (e.g. ampicillin) to determine the resistance prevalence for commensal *E. coli.* For aminoglycosides, that have a broad range of distinctly derived substances (Zembower et al., 1998; Fluit et al., 2001), two substances (gentamicin, streptomycin) were included in the resistance studies while antimicrobial use study presented data for the overall use of aminoglycosides.

In the ESVAC report, chloramphenicol was not included among the amphenicols as its use has been banned for use in food-producing animals in Europe since 1994 (European Commission, 1994). Nevertheless, chloramphenicol was the representative agent of amphenicols used in resistance studies and as such we justified direct comparisons between the two datasets.

Data analysis

Antimicrobial resistance prevalence was measured for each animal category. Exact binomial confidence intervals were calculated (with 95% confidence level) by means of a computed statistical algorithm (Pezullo; Clopper and Pearson, 1934). For further statistical analysis the antimicrobial resistance prevalence data were transformed using the arcsin transformation so as to follow bivariate normal distributions more accurately. Subsequently, for each antimicrobial class studied, the best fitting function describing the link between the use per country (expressed in mg/PCU) and the overall resistance prevalence of *E. coli* isolates for that specific antimicrobial agent was

determined and plotted. The coefficient of determination (R^2) was used to describe the proportion of variation explained by the function.

Additionally, each country was ranked (from lowest=1 to highest=7) in terms of resistance percentages on the one hand and use on the other hand for every antimicrobial agent studied seperately and the correlation was determined by means of the Spearman's rank correlation statistics (ρ), a nonparametric measure of statistical dependence between two variables. Finally, the average ranking of every country was also calculated for antimicrobial use (average of use ranks for all antimicrobial classes) and resistance (average of resistance ranks for all antimicrobial agents) and again the Spearman's rank correlation coefficient was determined.

Data manipulations and analysis were performed in Microsoft Excel© 2010 edition and IBM© SPSS Statistics 21.0.

Results

Veterinary antimicrobial use in Europe: the 2012 ESVAC report

The results from the seven selected European countries on antimicrobial use are presented graphically in Figure 1 for every antimicrobial class. It can be observed that total antimicrobial use differed between countries. Also, there is huge variation in amounts (mg/PCU) used of the different antimicrobial classes. For some classes this varied between 0 mg/PCU to 0.54 mg/PCU (third-generation cephalosporins) whereas for other classes this varied between 0.05 mg/PCU up to 74.46 mg/PCU (tetracyclines). Among countries, Belgium ranked first for six out of seven antimicrobial classes included. The Netherlands ranked first in tetracycline use.

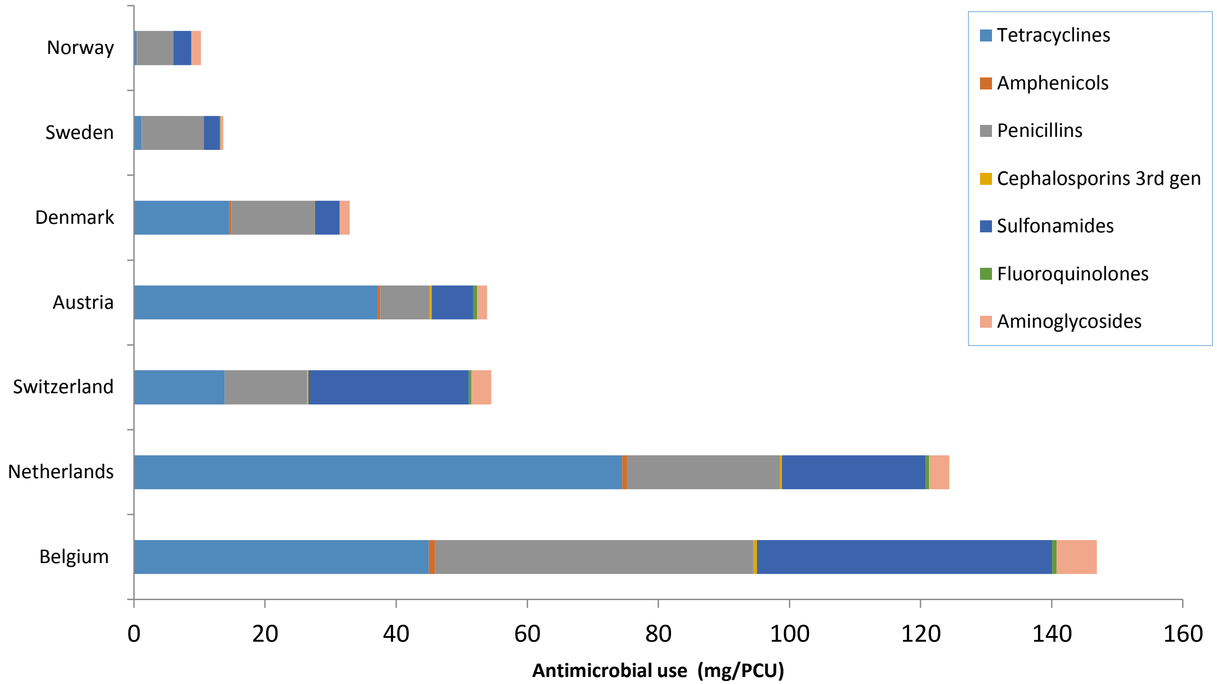

Figure 1. Antimicrobial use (expressed in mg/PCU) in food producing animals, for all countries and antimicrobial classes that were included in this study in 2010. Data from ESVAC report, 2nd edition[28]

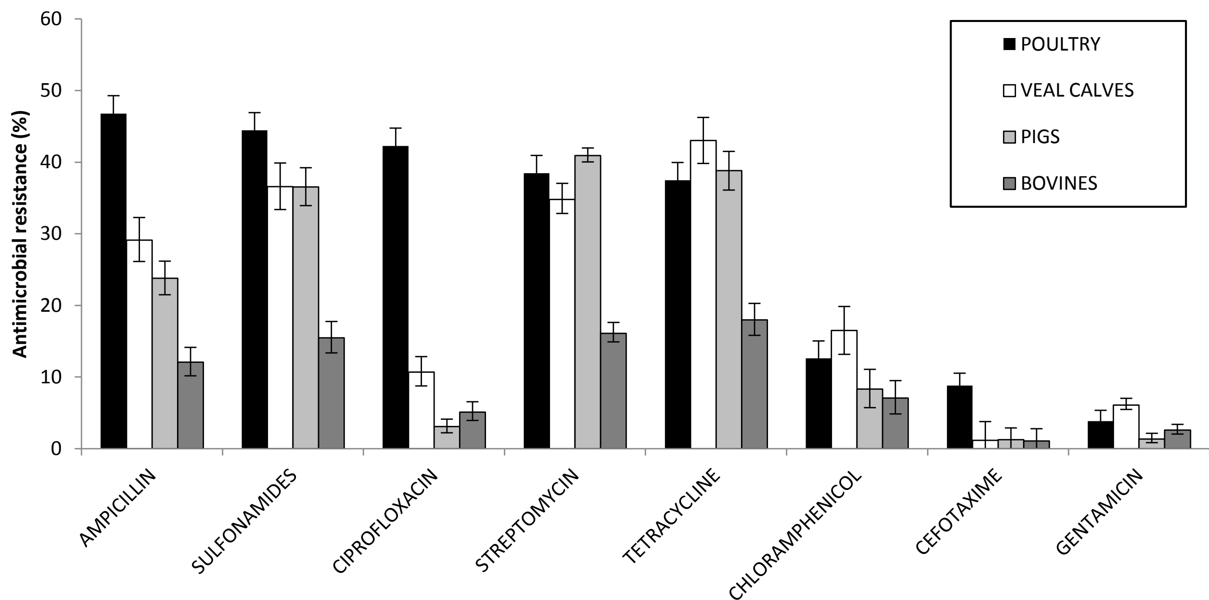

Figure 2. Prevalence of antimicrobial resistance in commensal *Escherichia coli* attributed to included antimicrobial agents, for the selected countries. Exact binomial confidence intervals were calculated with 95% confidence level. Data were compiled from various reports[19,20,22,27,44,45]

Antimicrobial Resistance in commensal Escherichia coli (data compiled from national reports)

In total, results on 4831 *E. coli* isolates were included. There were 1565 isolates retrieved from poultry, 1308 isolates from pigs, 1086 isolates from cattle and 872 isolates from veal calves. Huge variations in resistance levels were found both between animal species within countries and between countries within animal species. In general, resistance percentages were the highest for poultry isolates followed by veal calves, pigs and cattle (Figure 2).

Between countries, Belgium showed the highest levels of antimicrobial resistance for most antimicrobial agents studied in any animal category. For broilers, exceptions were gentamicin & chloramphenicol (Netherlands) and ciprofloxacin (Austria). For pigs, exceptions were tetracycline (Netherlands) and streptomycin (Austria). For cattle, exceptions were gentamicin and tetracycline (Netherlands). Last, for veal calves, exceptions were cefotaxime and streptomycin (Netherlands). Between animal species. *E. coli* isolates from broiler chickens showed the highest antimicrobial resistance prevalence for four antimicrobial substances (ampicillin, ciprofloxacin, streptomycin and cefotaxime). Veal calves isolates were the most resistant to tetracycline, chloramphenicol and gentamicin and isolates from pigs were most resistant for streptomycin.

Linking antimicrobial use and antimicrobial resistance

The quadratic regression function produced the highest R^2 values for all combinations of use data (mg/PCU) and resistance prevalence (Fig. 3).

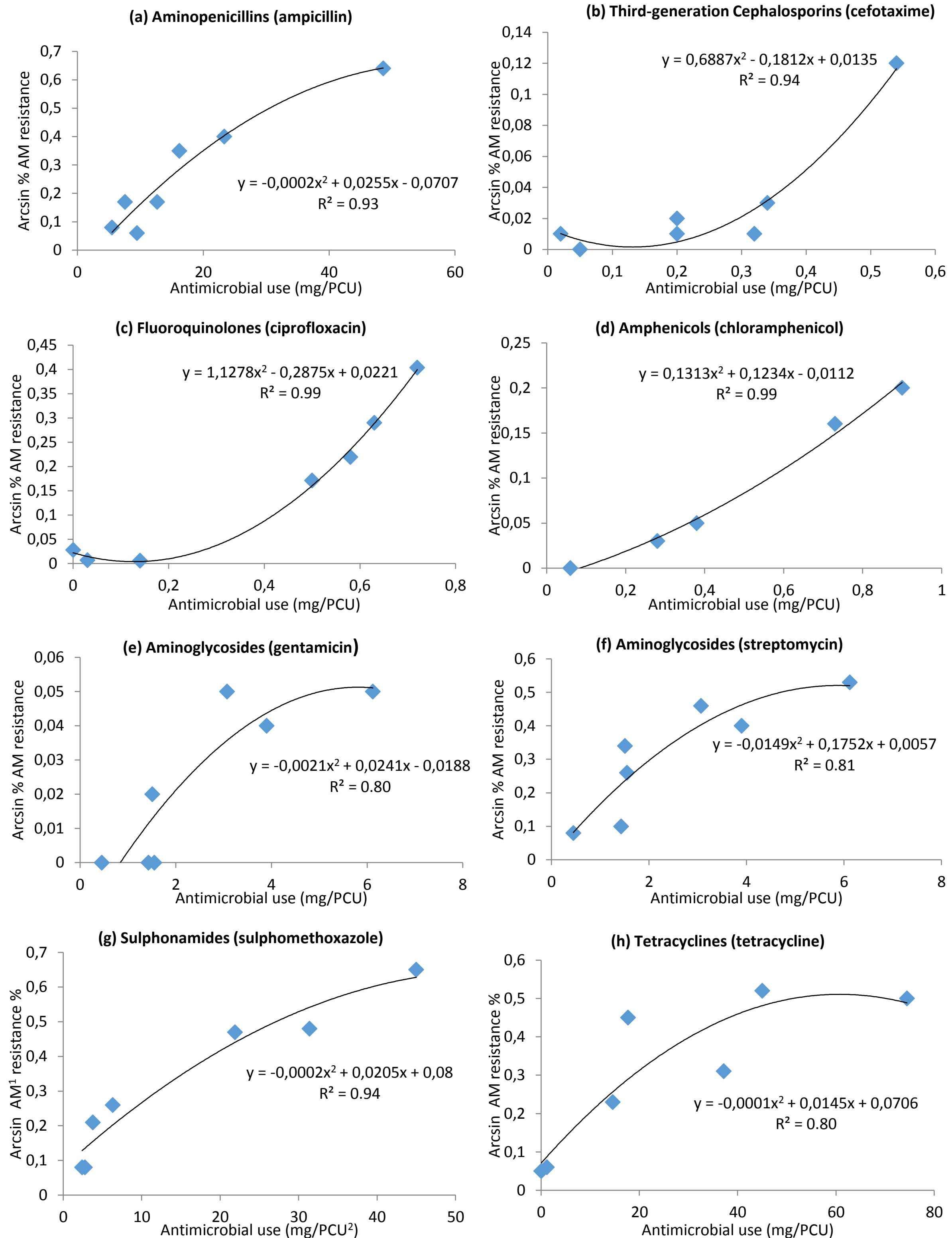

Figure 3 (a-h). Comparison between antimicrobial use data (expressed in mg/PCU[2]) to antimicrobial resistance prevalence data (arcsin transformed). A quadratic trendline was introduced. The equation used and the R-squared value are displayed. Data from seven European countries. [1] : AM =antimicrobial, [2] : PCU=population correction unit

High coefficient of determination values were observed in all studied classes with 0.99 for fluoroquinolones and amphenicols, 0.94 for third-generation cephalosporins and sulphonamides, 0.93 for aminopenicillins, 0.81 for streptomycin and 0.80 for gentamicin and tetracycline.

Table 1. Spearman's rank correlation coefficient measuring the correlation between antimicrobial use and antimicrobial resistance prevalence (arcsin transformed) for each antimicrobial class.

	Penicillins	Amphenicols	Third-generation cephalosporins	Tetracyclines	Fluoro-quinolons	Gentamicin	Streptomycin	Sulphonamides
Spearman's rank correlation	0.893	1.00	0.703	0.929	0.857	0.714	0.929	0.964
P-value	0.007	<0.001	0.078	0.003	0.014	0.071	0.003	<0.001

Each country was ranked (from lowest=1 to highest=7) in terms of resistance percentages and use for every antimicrobial agent studied separately and overall. Spearman's rank correlation coefficient was 1 for amphenicols, 0.96 for sulfonamids, 0.93 for streptomycin and tetracycline, 0.89 for aminopenicillins, 0.71 for gentamicin and 0.70 for third-generation cephalosporins (Table 1). The result of the overall ranking is shown in Figure 4.

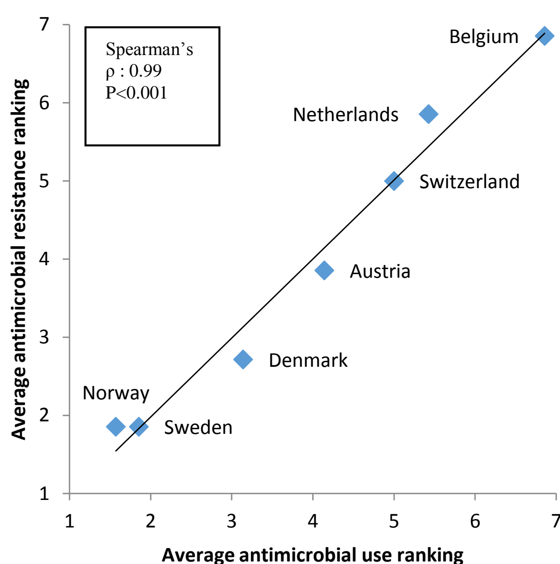

Figure 4. Spearman's rank correlation coefficient between average antimicrobial use ranking (lowest=1 to highest=7) of country and average antimicrobial resistance ranking (lowest=1 to highest=7) of indicator *Escherichia coli* isolates on all antimicrobial agents tested except amphenicols (not all countries provided usage data), for food producing animals. A dot represents the corresponding data of a single country. A linear trend line is introduced.

Discussion

In this study we attempted to link antimicrobial use data to antimicrobial resistance data at a national level. In order to do so we first had to compile available information on both variables.

The observed variation in antimicrobial use among countries is high. Of the seven countries for which sufficient data were available both on use and resistance, the lowest consumption for the antimicrobial classes used in this study was seen in Norway (10,22 mg/PCU) whereas the corresponding figure in Belgium is 146,9 mg/PCU (Figure 2) (ESVAC, 2012). When comparing the average use between the seven countries (62,83mg/PCU), the Netherlands' use is double the average use (125,85mg/PCU) in 2010-2011, while Denmark's use is approximately the half (33,23mg/PCU). Also among antimicrobial classes large differences were observed between countries. Already from 2010, Grave *et al* (2010) had concluded that there appears to be a wide variation between countries in the use of veterinary antimicrobial agents that cannot be explained by differences in the demographics of animal species. Differences in national policies on controlling antimicrobial use, veterinarians' prescribing and dosing habits, pharmaceutical marketing strategies, animal demographics and specific needs for antimicrobial use in specific countries related to specific diseases, can be possible explanations for the observed differences (ESVAC, 2012).

The antimicrobial resistance data also revealed variations between countries, between animal categories and between antimicrobial agents. Quinolones, an antimicrobial class that was introduced later than the other classes and was used extensively in poultry over the last decade (CODA-CERVA, 2012; Persoons et al., 2012), are now

already seriously compromised in broiler chickens. In Austria, resistance against ciprofloxacin is at an alarming point (80%). However, it should be noted that resistance in this paper is interpreted by EUCAST epidemiological cut-off values. The use of epidemiological cut-off values allows comparability over countries and offers the possibility of early detection of emerging resistance and is less subject to differences in opinion, which is often the case with more clinically orientated breakpoints (Cornaglia et al., 2004). To give an example, the ciprofloxacin resistance prevalence is not indicating a level of antimicrobial activity associated with a high likelihood of therapeutic failure (clinical resistance), but it is referring to the presence of acquired and mutational mechanisms of resistance to the agent (microbiological resistance). Nevertheless, as quinolones are a critically important antimicrobial class for human medicine, special actions should be taken for their use in animals.

In order to link antimicrobial use data to antimicrobial resistance data in veterinary medicine on a supra national level, we made use of publicly available information originating from national and international reports. Using this type of data had the significant advantage that available information was further explored and no additional expensive sampling and analysis were needed but also had a number of disadvantages in terms of data quality, availability and level of detail.

Where antimicrobial consumption is considered, an important data limitation is the fact that the antimicrobial use could not be estimated for each animal species. Silley *et al* (2012) stated that, ideally, for antimicrobial use data to have relevance to resistance-development patterns, these data should be recorded on the farm, along with the indication of treatment, the route of administration, the dose and duration of treatment and other relevant data, such as prevailing disease patterns and incidence. In a recent

study, Bondt *et al* (2012) demonstrated that the animal demographics strongly influence the antimicrobial use making overal comparisons based on mg/PCU difficult. In Denmark, detailed per species data are available (VetStat database), whereas for most other countries this is not yet the case (Stege et al., 2003). Such an advanced tracking system throughout the EU, would allow comparisons between countries and to antimicrobial resistance prevalence studies in more detail and with higher validity. In accordance, the recent initiative of ESVAC to move to antimicrobial use data collection at the species level is to be applauded (FVE, 2013). Another limitation is the use of mg/PCU as a consumption measure. Treatment frequency, using defined daily dose animal (DDDA) or used daily dose animal (UDDA), is a more refined measure of antimicrobial consumption (Callens et al., 2012; Persoons et al., 2012; Pardon et al., 2012). Although such data are not available at the moment on a European level, the decision of ESVAC to prioritise and suggest the use of DDDA and defined cure dose animal (DCDA) in the ESVAC project will surely provide in the future more detailed and accurate data (EMA, 2013). However, today these detailed and more accurate data sources are not available yet and therefore we used what is existing, taking into account the limitation.

Where antimicrobial resistance is concerned, *Escherichia coli* was selected as indicator bacterium for several reasons. First of all this indicator bacterium is the most used Gram-negative indicator bacterium (Asai et al., 2005; van den Bogaard and Stobberingh, 2000; Sorum and Sunde, 2001; Persoons et al., 2010), and therefore relevant data are available and secondly, the abundance of *Escherichia coli* in animal species and humans makes it one of the most likely vehicles for the spread of resistance genes (O'Brien, 2002). The limitation of this selection is of course the fact that the obtained results are only valid for this specific bacterium and cannot be readily

extrapolated towards other antimicrobial-bacteria combinations. Despite the selection criteria used to include resistance data, still large discrepancies between the number of isolates obtained per animal species and per country were encountered (Table S1). Bywater *et al* (2004) highlighted that, for surveillance studies performed at regular time periods, the number of samples are of great interest, so that the total number of samples give a representative image for the whole population. Thus, one challenge for future studies, would be to conduct them using representative populations.

A limitation of the data analysis is that correlations were studied between specific types of resistance and use without taking into account clusters of resistance (multi-drug resistance) in one isolate (data not available). As a consequence, the effects of co- and cross-resistance selection could not be estimated. Molecular epidemiology and characterization of resistance in strains would provide further insight in the complex mechanisms of antimicrobial resistance selection (Szmolka et al., 2012; Zou et al., 2012; Ewers et al., 2011).

Notwithstanding all the above mentioned limitations of the available data, it was deemed worthwhile to look at possible correlations between use and resistance at a meta level to determine whether the important differences in use between countries are in agreement with the reported differences in resistance. The coefficients of determination obtained through the quadratic trend lines were above 0.80 for all antimicrobial classes, suggesting that data on antimicrobial use is capable of explaining a large part of the variation observed in the resistance data at the national level. For most of the antimicrobial classes the best fitting curve describes an asymptotical form towards a certain maximum, translated into a negative sign of the quadratic part of the function. This suggests that for these antimicrobials, past a certain

antimicrobial use threshold, a further increase in antimicrobial use does not result in a further increase in the resistance percentage. Only for third-generation cephalosporins, amphenicols and fluoroquinolones a positive quadratic term is obtained resulting in an exponential curve suggesting that a small increase in use has a large increase in resistance as a consequence.

Also high correlations were found between the antimicrobial use and the antimicrobial resistance. For all antimicrobial agents, Spearman's rank correlation coefficient exceeded 0.70, indicating that in countries with high use of any of the selected antimicrobials also high levels of resistance are encountered (Table 1, Fig. 4).

Although all these observations are consistent with a link between use and resistance selection, we have to bear in mind that these analyses are performed on small datasets (7 datapoints per antimicrobial class) and therefore some caution is warranted in interpreting them. Moreover, the data did not allow for correction for the presence of clusters of resistance present in one strain and therefore the obtained correlation coefficients might be both over or underestimations of the true relationships between the use of specific antimicrobials and specific resistances. On the other hand, each of the datapoints in itself is a summary of a lot of information on antimicrobial use and resistance for each country. Looking at the information from such a meta point of view may allow to better recognize the overall relationships. Nonetheless more research, using more detailed data that will become available in the near future, should be performed to test whether the results obtained in this study can be confirmed.

Conclusions

The current paper, for the first time, describes the direct correlation of antimicrobial use data to antimicrobial resistance data in veterinary medicine on a supra national level based on publicly available data sources. The observed limitations in data and subsequent analysis restraints, reveal the need for further detail in collection and use and harmonization in resistance data collection in Europe. Despite these limitations, this comparison revealed high correlations for all antimicrobial classes studied. Bearing in mind that antimicrobial resistance is a global concern, the need for policies promoting lesser and more controlled use of antimicrobials is urgent and support for implementation should be provided on a European, or even better, global level.

Supplementary material

Table S1. Isolation fraction[a] of *Escherichia coli* isolates obtained to the overall number of isolates -per animal species

Country	Poultry	Pigs	Bovines	Veal Calves
Austria	0.11	0.13	0.17	-
Denmark	0.08	0.12	0.10	0.11
Netherlands	0.18	0.22	0.40	0.39
Belgium	0.27	0.12	0.14	0.04
Switzerland	0.12	0.14	-	0.21
Sweden	0.12	0.13	-	0.26
Norway	0.13	0.15	0.19	0.00

[a]It is the fraction of separate number of isolates presented at a national report divided to the total number of –per animal species - isolates from all the national reports studied in this article

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CHAPTER 4.

Chapter 4. Antimicrobial resistance prevalence among pathogenic and commensal *Escherichia coli* from food-producing animals in Belgium

Antimicrobial resistance prevalence among pathogenic and commensal *Escherichia coli* from food-producing animals in Belgium

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Abstract

Detailed studies on antimicrobial resistance for commensal *E. coli* (four animal categories) and pathogenic *E. coli* (pigs and bovines) in Belgium are presented for the year 2011 (www.coda-cerva.be). Poultry and veal calf isolates of commensal *E. coli* demonstrated higher antimicrobial resistance prevalence than isolates from pigs and bovines. Fifty percent of poultry *E. coli* isolates were resistant to at least five antimicrobials, whereas sixty-one percent of bovine *E. coli* isolates were susceptible to all antimicrobials tested. On the other hand, bovine pathogenic *E. coli* isolates showed an extended resistance profile with more than half of the isolates being resistant to ten or more antimicrobials. The results are not significantly different from the results from previously presented studies on commensal bacteria of pigs (Callens et al., 2010) and broilers (Persoons et al., 2010) in Belgium, although different methodologies of sampling and susceptibility testing were used.

Introduction

E. coli is often used as an indicator bacterium for the presence of antimicrobial resistance in the Gram negative bacteria because it is present in nearly all animal species. Murray et al. (1992) stated that resistance in commensal *E. coli* is an indication for the magnitude of the selective pressure from use of antimicrobials in an animal population. Some *E. coli* strains are also major pathogens in several animal species. In pigs, several studies indicate that antimicrobial resistance is higher in pathogenic than in commensal *E. coli* strains (Boerlin et al., 2005, Hendriksen et al., 2008).

Transfer of antimicrobial resistance from food-producing animals to humans might happen via food, through environmental contamination such as recreational waters and by direct animal contact (Wooldridge 2012). Infections with bacteria which are resistant to the antimicrobial used may result in treatment failures. Multi-resistance may necessitate the use of second-line antimicrobials for therapy, increasing the expenses as well as the chance of creating multi-resistant strains (Migliori et al. 2007). The World Health Organization (WHO) identifies antimicrobial drug resistance as a global concern and highlights the role of monitoring programs to provide sufficient data for use in ongoing research focussing at combatting drug resistance. To stimulate the discussion and the research for antimicrobial resistance in veterinary medicine, McEwen et al. (2002) stated that while antimicrobial resistance is also a major concern for animal health, yet little is known about the magnitude of this problem.

Given the importance of antimicrobial resistance, the Belgian Federal Agency for the Safety of the Food Chain has established a monitoring program for antimicrobial resistance in indicator bacteria. The monitoring in commensal *E. coli* complies with the guidelines set by EFSA. This monitoring program is the start of an annual returning

program which will allow to monitor the evolution of antimicrobial resistance and to evaluate the effect of intervention measures taken. It also allows the comparison of national results with other European countries (EFSA, 2012) or even beyond Europe. Until recently, there was also a monitoring on antimicrobial resistance in pathogenic *E. coli* from pigs and bovines at the Veterinary Agrochemical Research Centre (CODA-CERVA / VAR).

The aim of this study is to describe and summarize the results on *E. coli* of the official monitoring program and to compare them with data from other point prevalence studies on *E. coli*. For all the comparisons made between different studies of which the raw data were available,, the same interpretative criteria were applied (CLSI clinical breakpoints) to enhance comparability.

Materials and Methods

Monitoring of antimicrobial resistance in commensal E. coli from poultry, pigs, meat-production bovines, veal calves

All used sampling and analysis procedures are described in detail in the CODA-CERVA report on monitoring of antimicrobial resistance in *E. coli* in Belgium in 2011 (VAR, 2012). Briefly, faecal samples were collected by the inspectors of the FAV-AFSCA from randomly selected apparently healthy animals belonging to different categories (broiler chickens (n=420), pigs (>3 months old, n=157), meat-production bovines (>7 months old, n=154.) and veal calves (<7 months old, n=34) during 2011. The sampling ratio was one sample per farm. Isolates were identified as *E. coli* by Animal health care Flanders (DGZ) (inoculation on Kligler and indol medium) and the Walloon Regional Association for the health and identification of animals(ARSIA) (OPNG test, Ureum test and indol test) and then sent to the CODA-CERVA reference

laboratory for antimicrobial resistance where antimicrobial susceptibility was tested using a micro-dilution broth method (Trek Diagnostics©). The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration by which no visible growth could be detected. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut off values were used to indicate breakpoints for resistant or susceptible isolates on fourteen antimicrobial agents, as laid down by the European Commission.

Monitoring on antimicrobial resistance in pathogenic E. coli from pigs and bovines

All used sampling and analysis procedures are described in the CODA-CERVA report on pathogenic agents in Belgium (CODA-CERVA, 2012). Briefly, strains originating from diseased animals (pigs or bovines) that showed symptoms compatible with an *E. coli* infection were isolated and identified at the species-level. The bovine strains (all isolated from animals less than two weeks old) were selected on the basis of the presence of adhesion factors F17 and CS31A (by agglutination), tellurite resistance and enterohaemolysin production. Thus, most strains having no virulence factors were ruled out. Multiplex PCR tests were performed for pathotyping (Pigs: F4, F5, F6, F18, F41, Sta, Stb, LT, Stx2; Bovines: CNF1, CNF2, eae, vt1, vt2, Sta, F5, F17, F41).

The antimicrobial susceptibility was measured using the Kirby-Bauer disk diffusion method (NeoSensitabs, Rosco© tablets) and determined according to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines, and the CLSI clinical breakpoints. For pigs, fourteen antimicrobials were used, while for bovines twenty-four different antimicrobials were tested for. Data were interpreted as susceptible, intermediate resistant and resistant. The intermediate resistant strains were re-classified as resistant.

Study comparisons: data preparation and considerations

Regarding broiler chickens, the results of the national monitoring data were compared with the data obtained by Persoons et al. (2010) who determined the susceptibility of commensal *E. coli* from faecal samples from healthy broilers. For determination, they used the Kirby-Bauer disk diffusion method (NeoSensitabs, Rosco© tablets), and the determination of antimicrobial resistance was done according to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines. In Persoons *et al.* study faecal samples originating from 32 randomly selected broiler farms (30 samples per farm), between April 2007 and March 2008, were investigated. All farms were visited twice (two sampling periods). We compared only one sampling period (the second) so that all animals compared in this paper were sampled only once. From this sampling round, 912 strains were isolated. In order to make the comparisons valid, the raw data of the national monitoring on commensal *E. coli* in broilers were interpreted using the CLSI clinical breakpoints as used in the study by Persoons et al..

Results of the national monitoring on antimicrobial resistance in commensal *E. coli* from pigs were compared to the results on commensal *E. coli* in pigs by Callens et al. (Callens et al., 2010) who used Kirby-Bauer disk diffusion method (NeoSensitabs, Rosco© tablets), and the antimicrobial resistance prevalence was determined according to the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines. In this study, 824 strains originating from 45 Belgian randomly selected pig farms were tested (20 samples per farm). All animals were tested once. Similarly to the data of broilers, the raw data of the national monitoring on commensal *E. coli* in pigs were interpreted using the CLSI clinical breakpoints. We also compared the data on antimicrobial resistant pathogenic *E. coli* with those of the national monitoring on commensal *E. coli*, using the CLSI breakpoints.

Also, for fluoroquinolones - a critically important class of antibiotics for human medicine, there was a differentiation between studies. Both in the Persoons et al. study and in the Callens et al. study enrofloxacin was used, whereas ciprofloxacin was used in the national monitoring report. All fluoroquinolones have the same mechanism of action, that is inhibition of the expression of the topoisomerase genes leading to inhibition of DNA replication (Hopkins et al., 2005). As there is full cross resistance between fluoroquinolones, we compared directly the enrofloxacin with the ciprofloxacin resistance prevalence.

Data analysis

Antimicrobial resistance prevalence was measured for each animal category. Exact binomial confidence intervals were calculated (with 95% confidence level) after using a computed statistical algorithm (Pezullo, 2013). Significance of the standardized differences was tested using the Pearsons chi-square test.

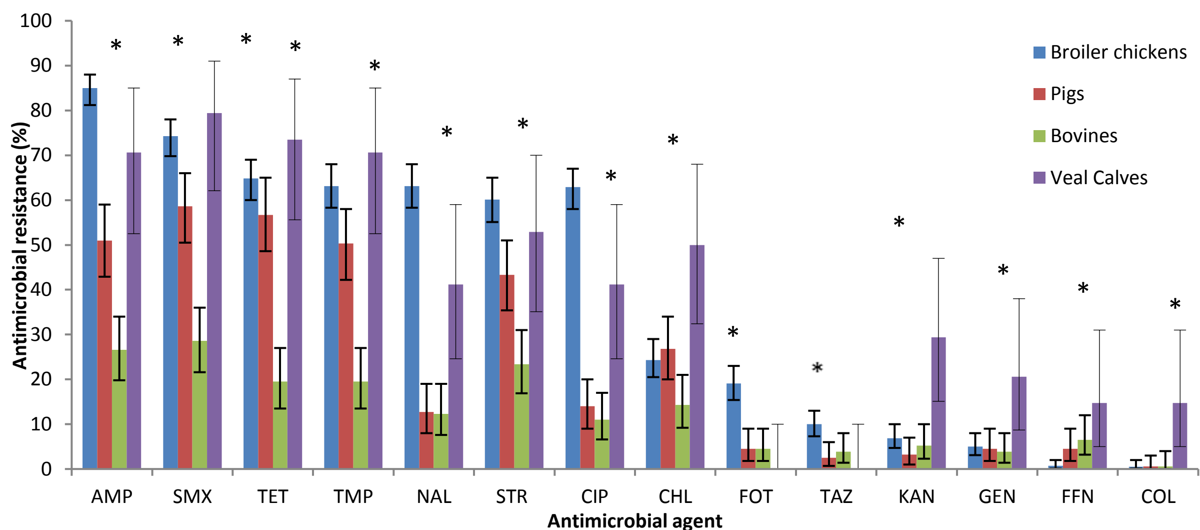
Results

National monitoring on antimicrobial resistance in commensal E. coli

In this study, 420 isolates from broiler chickens, 157 from pigs, 154 from meat-producing cattle and 34 from veal calves were collected. Antimicrobial resistance results are presented for every animal category in Figure 1.

For broiler chickens, antimicrobial resistance rose above 80% for ampicillin. For nalidixic acid, ciprofloxacin, sulphomethoxazole, tetracycline, streptomycin and trimethoprim the antimicrobial resistance prevalence was higher than 60%, but lower than 80%. Also, to be noticed are the 19.1% and the 10% antimicrobial resistance prevalence for the cephalosporins cefotaxime and ceftazidime, respectively. All ceftazidime resistant strains were also resistant to cefotaxime, as expected. The data

indicate that nearly half of these strains is carrying an ESBL gene and the other half a AmpC (eventually in combination with an ESBL) gene. Confirmatory testing is necessary to determine the full phenotype. For pigs, resistance prevalence was above 50% for ampicillin, sulphomethoxazole, tetracycline, and trimethoprim. For bovines, the highest antimicrobial resistance prevalence was seen for sulphonamides and ampicillin, with a resistance prevalence of approximately 25%. Concerning veal calves, resistance prevalence was higher than in pigs and bovines. More than 70% of the strains were resistant against ampicillin, sulphonamides, tetracycline and trimethoprim. Yet, no cephalosporin resistance was found in veal calves.



AMP: ampicillin, CHL: chloramphenicol, CIP: ciprofloxacin, COL: colistin, FFN: florfenicol, FOT: cefotaxime, GEN: gentamicin, KAN: kanamycin, NAL: nalidixic acid, SMX: sulfomethoxazole, STR: streptomycin, TAZ: ceftazidime, TET: tetracycline, TMP: trimethoprim

Figure 1. Indicator *Escherichia coli* isolates' resistance prevalence for fourteen antimicrobial agents. Confidence intervals are included. Four animal populations were studied. Micro broth dilution method was used and epidemiological cut off values were applied to determine the antimicrobial resistance prevalence, according to EUCAST standards. Significant differences between studies are indicated with * (P-value was set at 5%)

Table 1. Commensal Escherichia coli strain antimicrobial susceptibility rate, multi-resistance and main findings for each animal population included

Animal population	Strain susceptibility rate[a]	Multi-resistance median[b]
Broiler chickens	6.2 %	5 AMs[c] (6.5 for ESBL suspected strains, 8 for AmpC suspected strains)
Pigs	22.3 %	3 AMs
Bovines	61 %	0 AMs (5 for cephalosporin resistant strains, 5 for FFN resistant strains)
Veal Calves	14.7 %	5 AMs (7 for colistin resistant strains)

[a] : It is the percentage of the strains that remained fully susceptible to all antimicrobials
[b] : It is the modal number of antimicrobials to which 50% of the strains were resistant
[c] : AMs = Antimicrobial agents,
FFN=florfenicol

Multi resistance median and strain susceptibility rates are presented in Table 1. For broiler chickens and veal calves, more than 50% (multi-resistance median) of the *E. coli* strains acquired resistance to at least five antimicrobials. For pigs, the multi-resistance median was three and for bovines, more than 50% of the strains were fully susceptible to all antimicrobials. When viewing isolates from broiler chickens in more detail, for ESBL suspected strains the multi-resistance median was 6.5 antimicrobials and for AmpC suspected strains the median was eight antimicrobials.

Monitoring of pathogenic E. coli from pigs and bovines

A total of 135 pig strains were retrieved, of which 133 were analysed for virulence characteristics by PCR. In the majority of them (76/133), no virulence genes could be detected. The most prevalent pathotype was ETEC (Table 2). Few strains were positive for F41, F5 or F6 fimbriae. F4 was the most prevalent adhesion factor, followed by F18. Of the ETEC associated toxins, STb was the most prevalent. Haemolysis was seen in approximately 60% of the strains. Nearly 90% of the pathogenic strains were haemolytic. For bovine strains, 545 were obtained and the vast majority of them

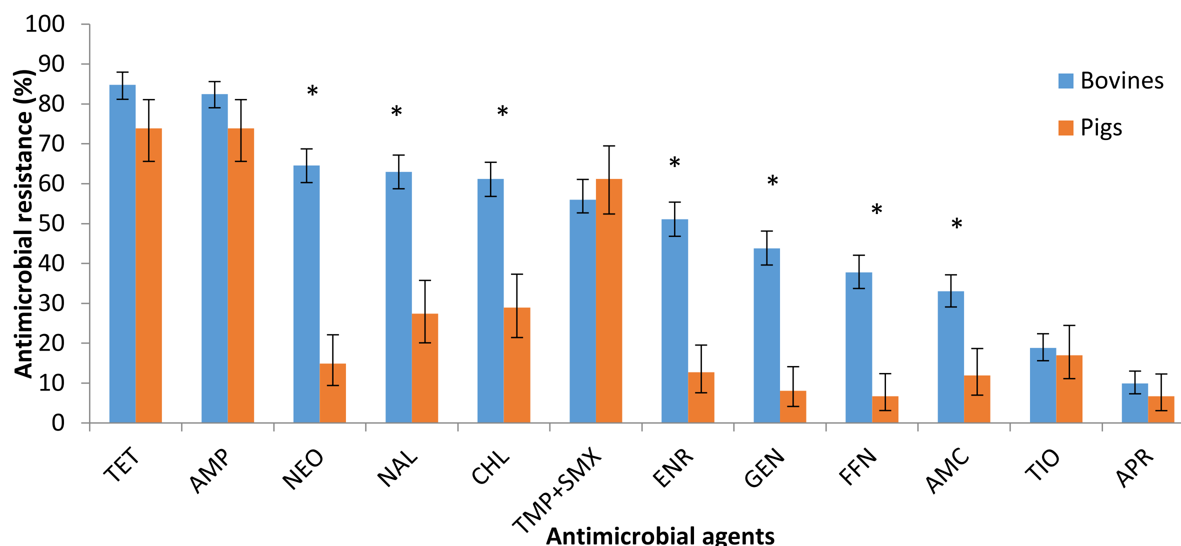
(468/565) were colonizing strains (Table 2). Six out of the 10 VTEC strains were associated with VT1, three with VT2 and one with both VT1 and VT2. EPEC strains were associated with F5 and/or F41. The majority of colonizing strains was CS31A or F17 positive.

Table 2. Pathotypes found in pathogenic *E. coli* strains from pigs and bovines in 2011

Pathotype	Number of strains (pigs)	Number of strains (bovines)
Colonizing strains	5	468
ETEC	35	11
VTEC	8	10
ETEC/VTEC	1	1
ETEC without attachment factor	8	0
No virulence gene/ factor detected	76	35
No final conclusion possible[1]	-	5
Total number of strains	133	545

[1]: this applies only for bovine *E. coli* strains due to the different pathotyping technique used

Antimicrobial resistance prevalence for pigs and bovines are shown in Figure 2. The multi-resistance median for pigs was five out of 15 antimicrobials and the strains that were fully susceptible were 7.4%. For bovines, the multi-resistance median was ten out of 24 antimicrobials, and the strains that remained fully susceptible to all antimicrobials were 3.9%.



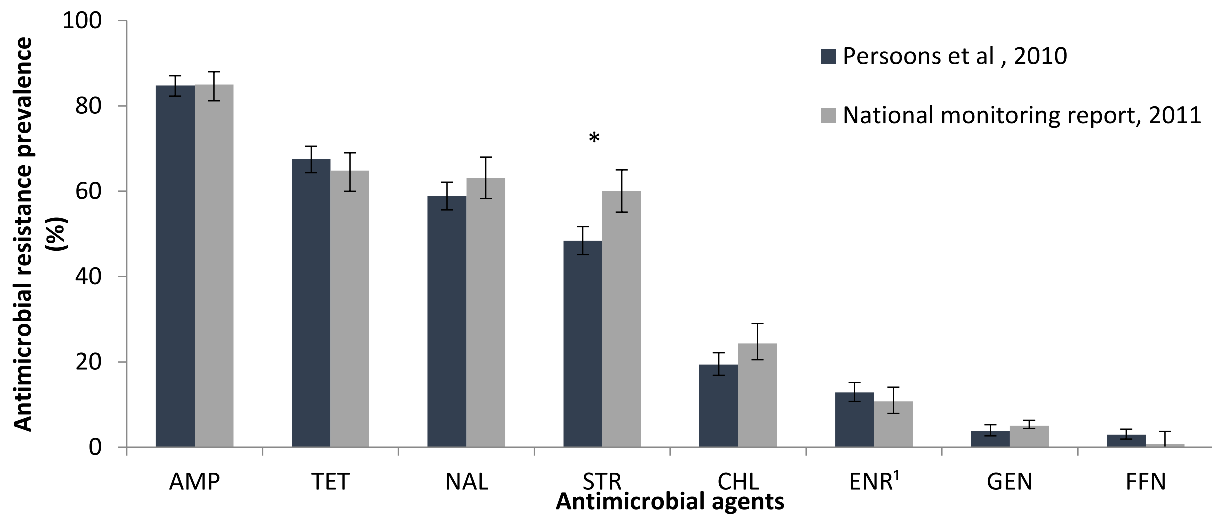
AMP: ampicillin, AMO-CLA: amoxycillin- clavulanic acid, TET: tetracycline, TMP: Trimethoprim, SUL: sulfonamide, TIO: ceftiofur, NAL: nalidixic acid, ENR: enrofloxacin, APR: apramycin, NEO: neomycin, GEN: gentamycin, CHL: chloramphenicol, FFN: florfenicol

Figure 2. VAR report, pathogenic *E. coli* antimicrobial resistance prevalence in pigs and bovines, in 2011. Additionally, confidence intervals were calculated. Disk diffusion method was used and clinical breakpoints (CLSI standards) were implemented. Note: only antimicrobial agents that were commonly tested in both animal species are displayed in this figure. Significant differences between studies are indicated with * (P-value was set at 5%)

Study comparisons

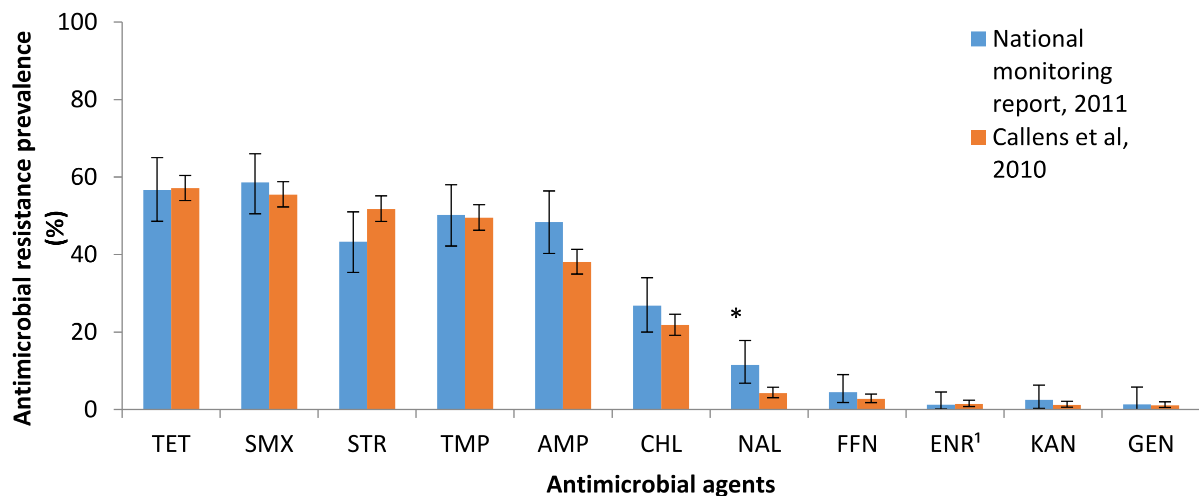
Comparing the data of the national monitoring on antimicrobial resistance of commensal *E. coli* from broilers with the data obtained in the study by Persoons et al. (2010) (Figure 3) reveals that only the resistance prevalence of streptomycin was significantly higher in the national monitoring report. Comparing the data of Callens et al. (2010) to the national monitoring in commensal *E. coli* from pigs (Figure 4), the only statistically significant difference found was for nalidixic acid, this being higher in the national monitoring report. Last, we compared the VAR commensal study and the pathogenic *E. coli* study for pigs (Figure 5) and bovines (Figure 6). For pigs, ampicillin, sulphamethoxazole, tetracycline and nalidixic acid showed a significantly higher antimicrobial resistance prevalence in the pathogenic *E. coli* study. For bovines, significantly higher prevalences were seen for ampicillin, sulphamethoxazole,

tetracycline, nalidixic acid, trimethoprim, streptomycin, fluoroquinolones, chloramphenicol, kanamycin, gentamycin and florfenicol.



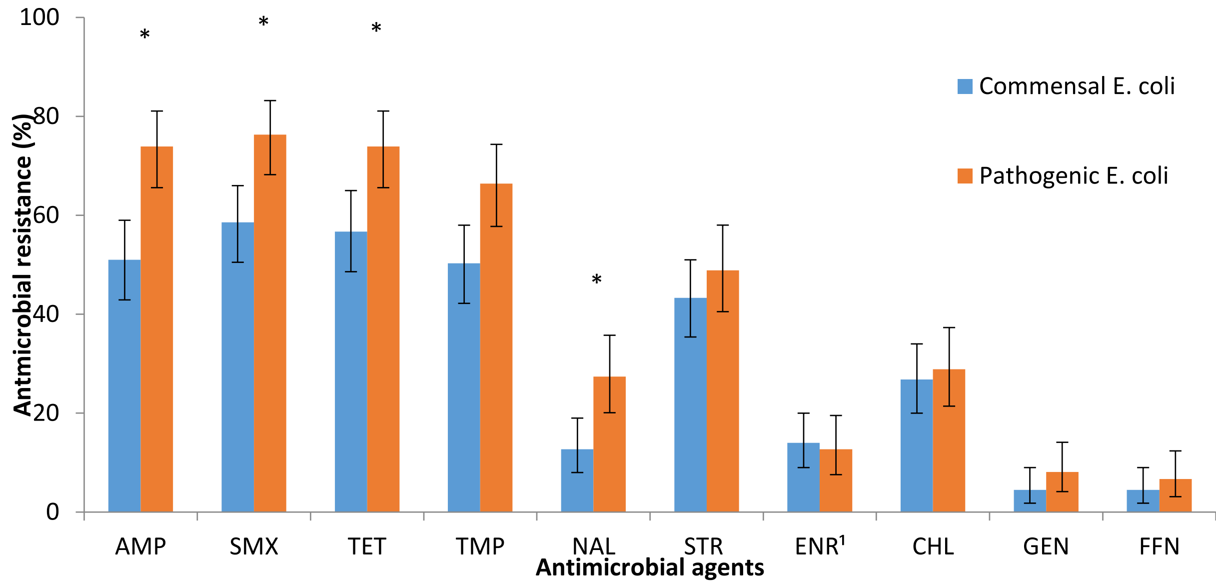
AMP: ampicillin, NAL: nalidixic acid, TET: tetracycline, STR: streptomycin, CHL: chloramphenicol, ENR[1]: enrofloxacin (national monitoring report used ciprofloxacin), GEN: gentamycin, FFN: florfenicol

Figure 3. Indicator *E. coli* resistance in poultry. Study comparison between VAR report and Persoons et al. study. Data harmonization with CLSI breakpoints for clinical resistance applied to both datasets. Additionally, confidence intervals were calculated. Significant differences between studies are indicated with * (P-value was set at 5%)



AMP: ampicillin, NAL: nalidixic acid, TET: tetracycline, STR: streptomycin, CHL: chloramphenicol, GEN: gentamycin, FFN: florfenicol, ENR[1]: enrofloxacin (national monitoring report used ciprofloxacin), SMX: sulfomethoxazole, TMP: trimethoprim, KAN: kanamycin

Figure 4. Indicator *E. coli* resistance in pigs. Study comparison between VAR report and Callens et al study. Data harmonization with CLSI breakpoints for clinical resistance applied to both datasets. Additionally, confidence intervals were calculated. Significant differences between studies are indicated with * (P-value was set at 5%)



AMP: ampicillin, SMX: sulfomethoxazole, TET: tetracycline, NAL: nalidixic acid, STR: streptomycin, ENR[1]: enrofloxacin (national monitoring report used ciprofloxacin), CHL: chloramphenicol, GEN: gentamycin, FFN: florfenicol

Figure 5. Study comparison between the VAR pathogenic *E. coli* study and the VAR commensal *E. coli* study. Data were collected from pig strains. Data harmonization with CLSI breakpoints for clinical resistance was applied to both datasets. Additionally, confidence intervals were calculated. Significant differences between studies are indicated with * (P-value was set at 5%)

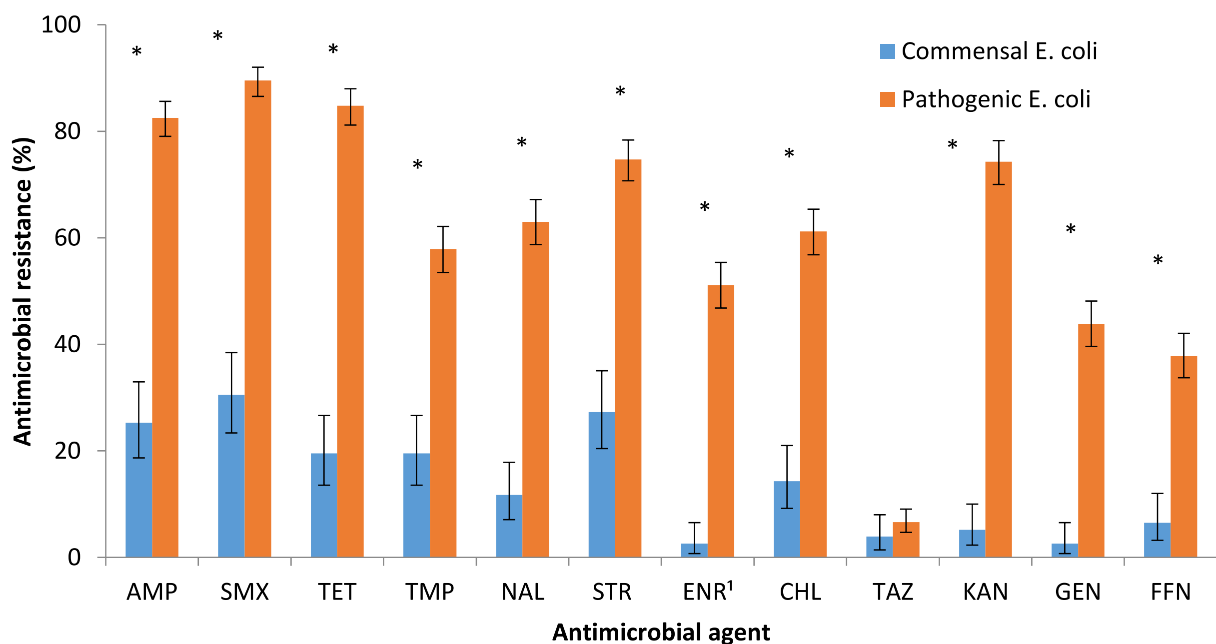


Figure 6. Study comparison between the VAR pathogenic *E. coli* study and the VAR commensal *E. coli* study. Data were collected from bovine strains. Data harmonization with CLSI breakpoints for clinical resistance was applied to both datasets. Additionally, confidence intervals were calculated. Significant differences between studies are indicated with * (P-value was set at 5%)

Discussion

Commensal E. coli

In the Belgian national monitoring program on antimicrobial resistance in commensal *E. coli*, samples were collected from broiler chickens, pigs, veal calves and bovines. Compared to other national monitoring programs conducted in European countries in 2010- 2011, this was the only report- alongside with the MARAN report- that included data from the four major animal categories, complied with the EFSA guidelines, and - at the same period- their countries provided detailed data on the sales of veterinary antimicrobial agents (Chantziaras et al., 2013). In addition, the number of samples taken was –with the exception of veal calves- comparable with other national monitoring reports conducted the same year, thus allowing to provide a representative overview of the resistance situation (Bywater et al., 2004). A further increase on the number of samples will nevertheless improve the power of the study for analysing trends on antimicrobial resistance.

When comparing the results between animal species for commensal *E. coli*, veal calf isolates showed the highest antimicrobial resistance prevalence for eight antimicrobial agents. However, the low number of veal calf samples that were included, resulted in large confidence intervals. Hence, when compared to the other animal categories studied, resistance prevalence results were not significantly different from broiler chickens or from pigs. Only when compared with bovines, and for all antimicrobials except cefotaxime and ceftazidime, resistance prevalence was significantly higher for veal calves isolates. Apart from that, the 14.7% antimicrobial resistance prevalence against colistin is to be noted. The colistin resistant strains were highly multi-resistant, all being resistant to at least seven more antimicrobial agents. Gram-negative bacteria can develop resistance to colistin through chromosomal mutation or adaptation

mechanisms rather than an horizontal spread of Mobile Genetic Elements (MGEs) carrying resistance genes (Falagas et al. 2005, Dai et al. 2008). In the latter, multi-resistance is of higher importance since co-resistance on eg. a same plasmid will select more for multi resistance. More recently, and after the description of plasmid-mediated colistin resistance through the transfer of *mcr* genes (Liu et al., 2016; Hasman et al., 2015; Torpdahl et al., 2017) it seems that these isolates contained such plasmids. To further support this claim, the findings from a paper published by Malhotra-Kumar et al. (2016) will be briefly presented. Malhotra-Kumar et al. screened a selection of 105 colistin-resistant *E coli* strains isolated during 2011–12 from passive surveillance of *E coli* diarrhoea in 52 calves from Wallonia and 53 piglets from Flanders, both regions of Belgium. All strains were screened for the presence of *mcr-1* genes. They detected *mcr-1* in six (11·5%) of the 52 strains were isolated from calves and seven (13·2%) of 53 were isolated from piglets.

Broiler chicken isolates showed the highest antimicrobial resistance prevalence for the other six agents. The antimicrobial resistance prevalence against quinolones was higher compared to the other animal species. Moreover, resistance to ceftazidime and cefotaxime was high, reaching a 20% prevalence for each. A further look into multi-resistance patterns of the isolates provided valuable information. All ceftazidime resistant strains were also resistant to cefotaxime. As shown in Table 1, there is a high multi-resistance pattern of the cephalosporin resistant strains. Due to the particular importance of cephalosporins for human health, molecular epidemiology analysis and further testing (*e.g.* detection of plasmid-mediated genes) of the strains in such studies is warranted. Samples from bovines were the least resistant against the antimicrobials used.

After comparing the national monitoring study for broilers with the results of Persoons et al. study, no significant differences were found with the exception of streptomycin resistance that showed a higher prevalence in the results of the national monitoring report. This difference could be attributed to the accuracy of disk diffusion method for streptomycin. In the study of Persoons et al. two sampling rounds were performed in total. We used the data of only one sampling round so that all animals in both datasets were tested only once. Apart from that, there was a disagreement concerning the number of samples obtained from each farm. We justified the comparison of the datasets, since in all farms the number of samples was steady (one sample per farm in the national monitoring report and thirty samples per farm in the Persoons et al. study). Comparing the national monitoring study for pigs and the respective study of Callens, only a small yet significant difference was seen for nalidixic acid, the prevalence of which was higher in the national monitoring report (Fig. 5). Contrary to the national monitoring study, all the studies that used the disk diffusion method did not test colistin as several papers have proved that the poor agar diffusion characteristics of colistin limit the accuracy of the disk diffusion test (Gales et al. 2001, Lo-Ten-Foe et al 2007, Galani et al. 2008). In general, the monitoring on commensal *E. coli* did not show many statistically significant differences between the national monitoring and the two selected point prevalence studies, both confirming the high antimicrobial resistance prevalence in Belgium. This agreement suggests that both the studies and the monitoring program were capable of describing the general level of resistance in a representative manner. It may therefore be concluded that the described resistance levels in commensal *E. coli* in pigs and broilers are truly the current level of resistance and are therefore a good reference point to check for evolutions in the coming years. Nevertheless, all the comparisons also revealed that

even when using different methods, a certain level of harmonization between *E. coli* studies can happen, acknowledging on beforehand the limitations that can be seen for some antimicrobials (e.g. colistin, streptomycin). Besides the methods used, special attention should also be drawn to the harmonization between sampling methods, age of animals, and number of samples.

Pathogenic E. coli reports

As shown in Figure 2, pathogenic *E. coli* strains from bovines are more resistant to antimicrobials than those from pigs. Antimicrobial resistance against florfenicol was five times higher in comparison to pigs, four times higher for neomycin and gentamicin and two times higher for chloramphenicol. No significant differences were seen for trimethoprim and its combination with sulphonamides, apramycin, ceftiofur and ampicillin. The use of the most recently introduced antimicrobials in veterinary medicine seems to be already compromised in pathogenic *E. coli*, especially for strains isolated from bovines. Regarding pigs, absence of haemolysis could be associated with the absence of virulence characteristics whereas haemolysis is not enough as a fast characterisation of virulence. The most multi-resistant strains (resistant against nine antimicrobials) were all ETEC strains. Regarding bovines, the strains in which no virulence factor was found, were the strains that were mainly selected on the basis of their positive tellurite reaction and haemolysis. Strains resistant against cephalosporins and strains resistant against amoxicillin with clavulanic acid were clearly associated with multi-resistance. Co-resistance with ceftiofur was seen for 23 cases suggesting the presence of CMY encoding genes or other genes. Further testing is warranted, as these antimicrobials are critically important for human and veterinary medicine.

Several authors (Boerlin et al., 2005, Hendriksen et al., 2008) have shown that antimicrobial resistance is more frequent in pathogenic than in commensal *E. coli* strains from pigs. For pigs, when comparing the commensal with the pathogenic *E. coli* VAR studies (Figure 5), pathogenic strains were significantly more resistant for four out of ten antimicrobials (ampicillin, tetracycline, sulphonamides and nalidixic acid). For the case of bovine strains (Figure 6), the differences between the antimicrobial resistance prevalence were more evident and more numerous (pathogenic strains were significantly more resistant for eleven out of twelve antimicrobials that were commonly tested). It should be mentioned that for the pathogenic *E. coli* studies, isolates were collected from clinical cases. Age of the animals, genetic background of the *E. coli* isolates, and the possible previous administration of antimicrobials to the clinically ill animals could explain partially these differences. Hence, when reviewing the observed differences in resistance rates between commensal and pathogenic *E. coli,* pathogenic isolates seem not to be the best choice for providing an overview on resistance levels and evolutions.

Conclusions

In 2011 a large scale national monitoring program on antimicrobial resistance in commensal and -alongside with the pre-existing program concerning zoonotic- bacteria was launched in Belgium. Antimicrobial resistance in commensal *E. coli* varied between animal species. Comparing results from commensal *E. coli* point prevalence studies in research projects, using a different sampling and susceptibility testing methodology, we revealed that results were highly comparable. Pathogenic *E. coli* strains both from bovines and pigs were more multi-resistant than the respective *E. coli* commensal strains.

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Chapter 5. Studying the effect of administration route and treatment dose on the selection of enrofloxacin resistance in commensal *E. coli* in broilers

Studying the effect of administration route and treatment dose on the selection of enrofloxacin resistance in commensal *E. coli* in broilers

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Abstract

Objectives: Factors potentially contributing to fluoroquinolone resistance selection in commensal *E. coli* strains in poultry were studied through a series of *in vivo* experiments. The effect of the initial prevalence of enrofloxacin resistance in the *E. coli* gut microbiota, the effect of the bacterial fitness of the enrofloxacin-resistant strain and the effect of treatment with enrofloxacin (effect of dose and effect of route of administration) were assessed.

Methods: Four in vivo studies with broiler chickens were performed. Right after hatching, the chicks were inoculated either with a bacteriologically fit or a bacteriologically non-fit fluoroquinolone-resistant strain either as a minority or a majority of the total *E. coli* population. Six days later, the chicks were treated for three consecutive days either orally or parenterally and using three different doses (under-, correct- and over-dose) of enrofloxacin. The faecal shedding of *E. coli* strains was quantified by plating on agar plates either supplemented or not supplemented with enrofloxacin. Linear mixed models were used to assess the effect of the aforementioned variables on the selection of enrofloxacin resistance.

Results: The factors that significantly contributed were treatment ($p<0.001$), bacterial fitness of the resistant donor strain ($p<0.001$), administration route ($p=0.052$) and the interactions between bacterial fitness and administration route ($p<0.001$).

Conclusions: In the currently used models, fluoroquinolone resistance selection was influenced by treatment, bacterial fitness of the inoculation strain, and administration route. The use of oral treatment seems to select more for fluoroquinolone resistance, especially in the model where a non-fit strain was used for inoculation.

Introduction

Antimicrobial resistance is a natural phenomenon dating back thousands of years before the use of antimicrobials (D'Costa et al., 2011; Wright and Poinar, 2012). Nevertheless, the use of antimicrobials has contributed to the rise of antimicrobial resistance in bacterial pathogens of human and veterinary importance (Snary et al., 2004; Soulsby, 2007; Wegener, 2003). Fluoroquinolones constitute a critically important class of antimicrobial agents that directly inhibit DNA replication and transcription. In veterinary medicine, fluoroquinolones are widely used, especially in broiler production (Gouvea et al., 2015), with the oral route being the preferred administration route. Despite their efficacy, the use of fluoroquinolones in veterinary medicine is controversial (Landoni and Albarellos, 2015). Concerns about the increasing resistance against enrofloxacin in poultry led to the withdrawal of its use in 2005 in the USA (FDA, 2005), while in Australia its use has never been authorized. Nonetheless, in two later studies from USA (Love et al., 2012) and Australia (Ingram et al. 2013), fluoroquinolone-resistant strains were detected in several broiler samples, prompting the authors to suggest either a non-proper enforcement of the ban (Love et al., 2012), or co-selection caused by the use of other antimicrobial agents (Ingram et al. 2013).

Several studies have suggested a link between oral treatment with antimicrobial agents and selection of antimicrobial resistance in chickens (Jurado et al., 2015; Kaesbohrer et al., 2012; Li et al., 2010; Simoneit et al., 2015). Nevertheless, only a few studies compare parenteral and oral treatment protocols in broilers, and they focus solely on the pharmacokinetics of enrofloxacin and not on its effect on resistance selection (Bugyei et al., 1999; Devreese et al., 2014). Whether the effect of all contributing factors could be quantified, the optimal regimen (dose, route of administration,

duration) could be improved to reduce resistance selection while maintaining clinical efficacy.

Antibiotic resistance mechanisms can induce a fitness cost to the bacterium. This cost is more considerable in chromosomal resistance mutations than in resistance acquired via horizontal gene transfer (Vogwill and MacLean, 2015). This cost is a key parameter in the spread and persistence of antimicrobial-resistant bacteria (Sandegren et al., 2008). However, it is not yet known to what extent fitness influences the resistance selection. Studies supporting the reversibility of antibiotic resistance through minimizing antimicrobial use are available (Andersson, 2006; Graesboll et al., 2014; Levin 2002; Levin et al., 1997; Levin et al., 2000), though other studies focus on concepts such as compensatory evolution and genetic co-selection that make reversibility less probable (even if a fitness cost is present) in real-life settings (Andersson and Hughes, 2010; Kunz et al., 2012; Sundqvist et al., 2010). The potential for reversing antibiotic resistance through the reduction of antibiotic use will be dependent on the fitness cost of the resistance mechanism, the epidemic potential of the bacteria, and the transmission route of the species (Sundqvist, 2014).

Overall, there is insufficient information on the epidemiology of antimicrobial resistance, and this lack hampers efforts to provide appropriate and specific advice on measures that might reduce risks of resistance selection. In response, the present study aims at quantifying the effects of different factors on fluoroquinolone resistance in commensal *E.coli* in broilers, using well-defined and controlled experimental *in vivo* models. Four *in vivo* experiments were designed to study the influence of the: i) prevalence and ii) fitness of enrofloxacin-resistant strains in the early (one-day-old) *E. coli* gut microbiota, iii) treatment dose and iv) route of administration of enrofloxacin on fluoroquinolone resistance selection in commensal *E. coli*.

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 (EC2014/141, EC2015/33, EC2015/34, EC2015/61).

Bacterial strains

E. coli IA2 strain was obtained from a faecal sample of a healthy broiler chicken. The isolate was confirmed as being *E. coli,* and after being serotyped with the following monospecific antisera against 24 different somatic O antigens: O1, O2, O5, O6, O8, O9, O11, O12, O14, O15, O17, O18, O20, O35, O36, O45, O53, O78, O81, O83, O102, O103, O115, O116, it was deemed to be non-serotypable (identified and serotyped by CODA CERVA, Uccle, Belgium). Moreover, the strain was susceptible to all tested antibiotics (Table S1). On top of that, no plasmid mediated quinolone resistance (PMQR) genes were detected after using a protocol described by Robicsek *et al.* to detect for any *qnrA, qnrB, qnrS* determinants (Robicsek et al., 2006b), and by Park *et al.* to detect for the *aac(6')-lb-cr* determinant (Park et al., 2006).

Using this strain, a spontaneous rifampicin-resistant mutant strain (*E. coli* IA31) was derived as previously described (Tóth et al., 2003). Using IA31 and the same technique, a bacteriologically non-fit (*E. coli* IA50) enrofloxacin-resistant strain and a bacteriologically-fit (*E. coli* IA66) enrofloxacin-resistant strain were created (Table 1). A few spontaneous nalidixic acid-resistant strains were obtained by inoculating 1ml of an overnight culture (on Luria Bertani (LB) broth) of the reference (nalidixic-acid-susceptible) strain on a McConkey agar plate supplemented with 16 µg/ml nalidixic

acid. After an overnight culture of these mutant strains (in LB broth), 1 ml of each culture was further inoculated on a separate McConkey agar plate supplemented with 0.25 μg/ml enrofloxacin. After separate overnight cultures (on LB broth) of the strains that were able to grow on the latter plates, 1 ml was further inoculated on a McConkey agar plate supplemented with 1 μg/ml enrofloxacin. In the same manner, after separate overnight cultures (on LB broth) of the strains that were able to grow on the latter plates, 1 ml was further inoculated on a McConkey agar plate supplemented with 4 μg/ml enrofloxacin. The strains that were able to grow on the latter plates, were isolated and after a separate overnight culture on LB broth, 1 ml of each culture was further inoculated on a separate McConkey agar plate supplemented with 8 μg/ml enrofloxacin. Isolates that grew on the latter plates were collected and tested for bacterial fitness. Bacterial fitness was assessed with *in vitro* growth competition assays between each resistant strain and the parental susceptible strain (Andersson and Hughes, 2010). Equal densities of the enrofloxacin-susceptible and the enrofloxacin-resistant strain were mixed and incubated in antibiotic-free LB medium. Every 24h, 0.05 mL of the overnight culture was inoculated into 5 mL of new LB medium for growth. Aliquot parts of the same volume were plated, via a spiral plating technique, every 24h onto drug-free McConkey agar to count the number of colonies and onto McConkey agar plates containing enrofloxacin 1 mg/L to count the number of enrofloxacin-resistant strains. The number of parental enrofloxacin-susceptible colonies was calculated as the total number of bacterial cells minus the number of drug-resistant bacterial cells. The relative fitness was calculated as described (Petersen et al., 2009). The relative fitness was calculated as $r= ln(rt/rt\text{-}1)/ ln(st/st\text{-}1)$, where *rt* and *st* denote the absolute number of drug-resistant and drug- susceptible cells at a given time t, respectively, and *rt-1* and *st-1* denote the number of drug-

resistant and drug-susceptible cells at the preceding time point. All experiments were performed in triplicate with three independent cultures and a weighted mean was used for analysis.

The MIC, MBC and mutant prevention concentration (MPC) of the reference and the resistant strains were determined as previously described (Haritova et al., 2006; Olofsson et al., 2006; Zhao and Drlica, 2001, 2002). For the determination of MPC, two hundred microliters of a concentrated cellular suspension, containing more than 10^{10} CFU/mL, were plated on each of three Mueller Hinton Agar (MHA) plates supplemented with enrofloxacin at various concentrations equal to 1, 2, 4, 8, 16, 32, 64 X MIC. starting for susceptible strain from 0.016 mg/L to 1mg/L and for resistant strains from 32 until 2048mg/L. Plates were incubated at 37 °C for 5 days and were inspected for the presence of colonies after 24 hours, 72 hours and after 5 days. The MPCs were recorded as the lowest drug concentration preventing the emergence of any mutants after 2 and 5 days incubation. Each experiment was carried out in triplicate with three independent cultures. *E. coli* ATCC 25922© was used as a control strain for the *in vitro* tests.

PCR amplification and DNA sequence analysis

For the PCR amplification and sequencing of *gyrA, gyrB, parC,* and *parE* genes, the respective primers were used (Everett et al., 1996; Vila et al., 1994; Weigel et al., 1998) (Table S2). The amplification protocol was performed with a MasterCycler Gradient EP-S Thermal Cycler (Eppendorf AG, Hamburg, Germany). The PCR products for strains IA31, IA50 and IA66 were sequenced (Eurofins Genomics GmbH, Ebersberg, Germany) and the nucleotide sequences obtained were analyzed for the presence of point mutations in the quinolone resistance determining regions (QRDR) using the

BLAST search engine and the ClustalW multiple alignment tool (NCBI, 2016) (Table 2).

In vivo trials

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 a gas formaldehyde mixture at the hatchery, but after transportation they were additionally dipped in 5% H_2O_2 for 10-15 seconds. After drying for 20-25 seconds, they were further incubated in three separate sanitized hatching cabinets. Each cabinet was placed in a separate decontaminated stable.

As soon as the chicks were hatched, they were orally inoculated (Table 1) and subsequently housed in groups (each group consisting of five chicks) in $1m^2$ disinfected boxes in HEPA-filtered stables. Nine groups were used in each experiment and in total, 180 chickens were used in this study (45 per experiment). The birds received 16 hours of light daily, and had free access to autoclaved food and bottled water. Each bird was individually numbered. All birds were clinically examined on a daily basis and any clinical signs of disease were registered. Euthanasia was performed by intravenous injection with an overdose (10 mg/kg) of sodium pentobarbital 20% (Kela, Hoogstraten, Belgium).

Experimental setup

As soon as the chickens were hatched, they were all inoculated with a specific bacterial inoculum in accordance with the experiment performed (Table 1). The experimental setup, treatment and sampling procedure was identical for all experiments (Figure 1).

All necessary biosecurity measures were taken to avoid any cross-contamination (feed, water, indirect contact, air-borne transmission) between groups. Each stable contained a control group (non-treated animals), a group that was treated with enrofloxacin (Baytril™ 2.5% inj. Solution, Bayer AG, Leverkusen, Germany) intramuscularly and a group that received enrofloxacin via drinking water (Baytril™ 10% oral solution, Bayer AG, Leverkusen, Germany). Water was provided *ad libitum*. Treatment period lasted three days (day 6 to day 8). Treatment doses (Table S3) were calculated on the basis of the daily average body weight and the average water consumption (Bayer Animal Health, 2016), although water intake was not measured in detail.

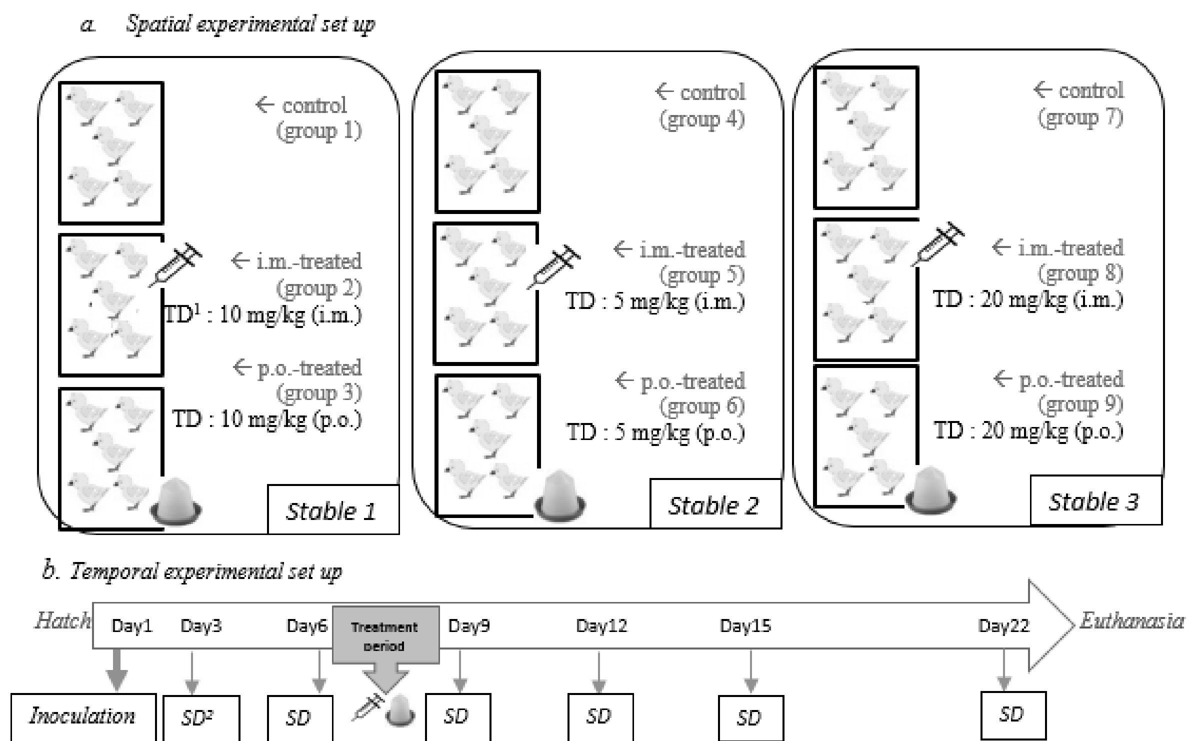

Figure 1. Schematic plan of the experiments (same for each experiment performed). As soon as the chicks were hatched, they were inoculated with a specific bacterial inoculum according to the experiment performed. Each stable contained a control group (non-treated animals), a group that was treated with Baytril™ 2.5% inj. solution intramuscularly (thigh muscles) and a group that received oral treatment (Baytril™ 10% oral solution). Treatment period lasted 3 days (day 6 to day 8). The treatment started right after the second sampling took place. The treatment dose schemes (Stable 1: proper-, Stable 2: half- and Stable 3:double- dose) were calculated based on the recommended therapeutic protocol of the company (Bayer AG, Leverkusen, Germany). The drinking water medication was prepared daily. one sampling every 3 days until day 15. Then, one additional sampling took place right before euthanasia on day 22.
[1] : Treatment dose, [2] : Sampling day

In total, six faecal samplings took place in each experiment. Starting from day 3 of the experiment, there was one sampling every 3 days until day 15. Then, one additional sampling took place right before euthanasia on day 22. Each sample was collected from a single animal and consisted of approximately 1gr of faecal content. Upon collection, all fresh individual samples were placed in sterile tubes and immediately transported to the laboratory for bacteriological enumeration.

Table 1. Strains (A.)and inoculums (B.) used in this paper. For all inoculums, the volume (dose per animal) was 0.2mL. and the concentration (cfu/mL inoculum) was ~ 10^8 cfu/mL.

A. Strain	Parental strain	Bacteriological fitness (compared to its parental strain)	Resistance against enrofloxacin	Resistance against rifampicin (marker)
E.coli IA50	*E. coli* IA31	Non-fit	Resistant	Resistant
E.coli IA66	*E. coli* IA31	Fit	Resistant	Resistant
E. coli IA31	*E. coli* IA2	Fit	*Susceptible*	Resistant

B. Inoculum	1	2	3	4
Used at	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Strains used	IA50 : IA31	IA66 : IA31	IA66 : IA31	IA50 : IA31
Ratio	100 : 1	100 : 1	1 : 100	1 :100

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 *E. coli* population (Eddy Jet, IUL Instruments, Barcelona, Spain).

All serial dilutions were plated on both i) rifampicin-supplemented (100 mg/L) McConkey agar plates and ii) enrofloxacin-supplemented (0.25 mg/L) and rifampicin-supplemented (100 mg/L) McConkey agar plates. Since the enrofloxacin-susceptible strain cannot grow on the enrofloxacin-supplemented plates, these plates were used

to differentiate the inoculated strains and allow for the calculation of the ratio of resistant strains .

After the grafting of each plate, they were placed in an incubator set at 37.0°C ± 1°C and examined after 24h ± 3h for the presence of typical colonies of *E.coli*. The colonies per plate were counted on plates ideally having 20 - 200 colonies per plate and the number of colony forming units (cfu)/g of faeces was calculated. In the exceptional case where less than five colonies per plate were counted in the lowest dilution, the sample was omitted.

Statistical analysis

In all the following analyses, linear mixed models were used (IBM SPSS Statistics for Windows, version 23.0, Armonk, NY). Each animal was listed as 'subject', and the sampling as 'repeat'. An autoregressive covariance matrix of the first order was used for the repeated covariance structure. To correct for the interdependency of chicks within a given pen, 'pen' was included as a random variable. The dependent variable used was the ratio of the number of enrofloxacin-resistant colonies to the total number of retrieved *E.coli*.

<u>Assessment of treatment</u>

The proportion of resistant strains over time was compared between animals that received treatment and those that did not. Observations for day 3 and day 6 (prior treatment) were similar within each respective experiment and in order to focus on the intervention effect of the treatment only observations from day 9 onwards (after treatment) were included.

Assessment of prevalence (inoculum ratio), fitness of enrofloxacin-resistant strains, treatment dose and route of administration

All animals that received treatment were included in the model. The fixed factors that were studied were the following: inoculum ratio (highly resistant, highly susceptible), bacteriological fitness (fit, non-fit), treatment dose (proper-, half-, double- treatment schemes) and route of enrofloxacin administration (oral, parenteral (i.m.)). All potential fixed factors were first tested univariately. Only variables with a P-value < 0.2 were selected to be included in a mixed (multivariate) linear model. The model was built according to a stepwise forward selection procedure. In the final mixed linear model, two-way interactions between significant variables were also evaluated. The main effects from the fixed factors were compared and Bonferroni correction was used to adjust confidence intervals. Throughout the entire analysis, the significance level was set at P ≤ 0.05.

Results and Discussion

In vitro characterization of strains

The DNA sequencing of the QRDR's of *gyrA, gyrB, parC and parE* are shown in Table 2. The reference strain showed no codon mutations resulting in amino acid changes when compared with *E. coli ATCC 25922*. For *gyrA*, two point mutations at codons 83 and 87 took place in both IA50 and IA66 when compared to the IA31 strain. Furthermore, for *parC*, a mutation at codon 78 occurred in the non-fit isolate, whereas a mutation at codon 80 in the fit strain was seen. In various studies (Johnning et al., 2015; Jurado et al., 2008; Lysnyansky et al., 2013; Morgan-Linnell et al., 2009; Vicca et al., 2007; Zayed et al., 2015), mutations in *parC* accompanied the mutations from *gyrA* in the vast majority of the clinically-resistant isolates that were tested. In these

studies the predominant mutation in *parC* was found in codon 80, accompanied in some isolates by a mutation in codon 84. When comparing the strains in these studies with the strains of this paper, the fit strain had a mutation in codon 80, while the mutation in the non-fit strain occurred in codon 78. To the authors' knowledge, only two strains with a mutation in codon 78 (for *parC*) have been reported previously in the literature (Heisig, 1996; Jurado et al., 2008), and for IA50, the exact combination of codon changes here reported is described for the first time. It is not clear whether these mutations could be linked with the strain's inferior bacterial fitness (Park et al., 2013). In an *in vitro* study in which the fitness of isogenic resistant strains was assessed (Marcusson et al., 2009), the strains with similar mutations with IA66 were also found to be bacteriologically fit when compared with their parental strains.

The enrofloxacin MIC levels of the isogenic resistant strains were both 32 mg/L, and for the (parental) susceptible strain 0.032 mg/L. Likewise, the MPC of enrofloxacin for the susceptible strain was 0.512 mg/L (corresponding to a 16-fold increase in comparison with the MIC), while the MPC for the resistant strains was 1024 mg/L (Table 2). In a study from Devreese *et al.* (2014), a validated liquid chromatography-tandem mass spectrometry method for the quantification of enrofloxacin in the intestinal content of broiler chickens was described. There it was shown that after the administration of 10 mg/kg enrofloxacin (p.o. and i.m.), the intestinal microbiota in cecum and colon was exposed to significant levels of enrofloxacin (21–130 µg/g). Therefore it can be assumed that in the *in vivo* experiments of the present study, the gut concentrations of enrofloxacin were much higher than the MPC of the susceptible strain. Although selection for additional resistance in the susceptible strain cannot be excluded, the observed changes in the proportions of susceptible and resistant strains were interpreted as being the result of the multiplication of the already present

(inoculation) resistant or susceptible stains. As a consequence, the effects described are caused by resistance selection rather than by the emergence of new resistance. This is further supported by the fact that the enrofloxacin resistance of the resistant strain is located in the chromosome (non-mobile), indicating that only the fluoroquinolone resistant strains were spreading under the selection pressure of the treatment (MIC value of 32 mg/L). Inclusion of a control group inoculated with a fully fluoroquinolone susceptible inoculum would have provided further information on the selection for additional resistance in the susceptible strain, but due to the limitations of the experimental setup, this was not feasible.

Table 2. In vitro results. The topoisomerase mutations, the MIC, MBC and mutant prevention concentration (MPC) are presented. E.coli ATCC 25922 was used as a control strain to identify any amino acid changes when compared to E. coli IA31.

		E. coli ATCC 25922[a]	*E. coli IA31*[b]	*E .coli IA50*[c]	*E .coli IA66*[d]
GyrA *changes*	Nucleotides	167542- 166980			
	Amino acid	5-191	NC[e]	83 (S->A (+)) 87 (D->G)	83 (S->L) 87 (D->G)
	Accession nr.[f]	ref|WP_001281242.1|	KX525205	KX525206	KX525207
GyrB *changes*	Nucleotides	853200- 853572			
	Amino acid	347-467	NC	NC	NC
	Accession nr	ref|WP_000072067.1|	KX525208	KX525209	KX525210
ParC *changes / Codon number*	Nucleotides	1589650 – 1589896		78: GGC -> GAC	80: AGT -> AGA
	Amino acid	53-133	NC	78 (G -> D)	80 (S -> R)
	Accession nr.	ref|WP_001281881.1|	KX525211	KX525212	KX525213
ParE *changes*	Nucleotides	1573843- 1574108			
	Amino acid	412-499	NC	NC	NC
	Accession nr	ref|WP_000195296.1|	KX525214	KX525215	KX525216
Enrofloxacin cut-off values	MIC (mg/L)	0.016	0.032	32	32
	MBC (mg/L)	0.016	0.047	64	32
	MPC (mg/L)	0.512	0.512	1024	1024

a: This is the in vitro control strain. All changes in the amino-acid level will be enumerated according to this strain b: This is the enrofloxacin-susceptible strain used in all in vivo experiments. c: Enrofloxacin-resistant non-fit mutant strain. d: Enrofloxacin-resistant fit mutant strain., e: NC: No changes took place. f: Accession number provided by GenBank (NCBI, Bethesda, MD, USA)

Phenotyping of E. coli from in vivo experiments

No animal showed any signs of disease throughout the duration of the experiments. The *E. coli* strains that were used, successfully colonized the gastrointestinal tract of the animals (Figures 2-5). In several studies examining the gastrointestinal microbiota of hatched chickens (Lu et al., 2003; Shaufi et al., 2015), it has been found that after reaching optimal growth within the first 2 days of life, the *E. coli* population decreased rapidly at 8-9 days and was hardly found at 14 days. Hence, while the very young chick is quite a good and permissive host for *E. coli* (Baron et al., 2016), it is quickly colonized by other types of bacteria. Although this decline was also observed in the present *in vivo* experiments (data not shown), a sufficient number of *E. coli* colonies were nevertheless retrieved in the majority of the samples until the end of the experiment, thus allowing for a full evaluation of the effect. Only in Experiment 2, on the last sampling day and for the samples from orally-treated animals (half dose group: 5mg/kg), were no *E. coli* cfu found on the McConkey plates (Figure 3).

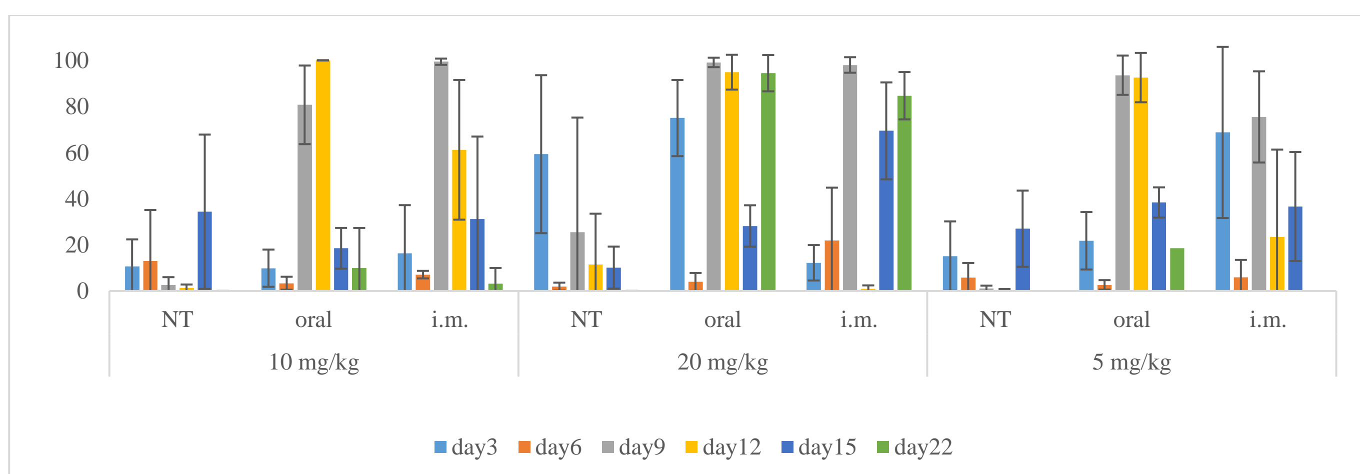

Figure 2. Experiment 1 (inoculation with a bacteriologically non-fit resistant strain and a bacteriologically fit susceptible strain in a 100:1 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

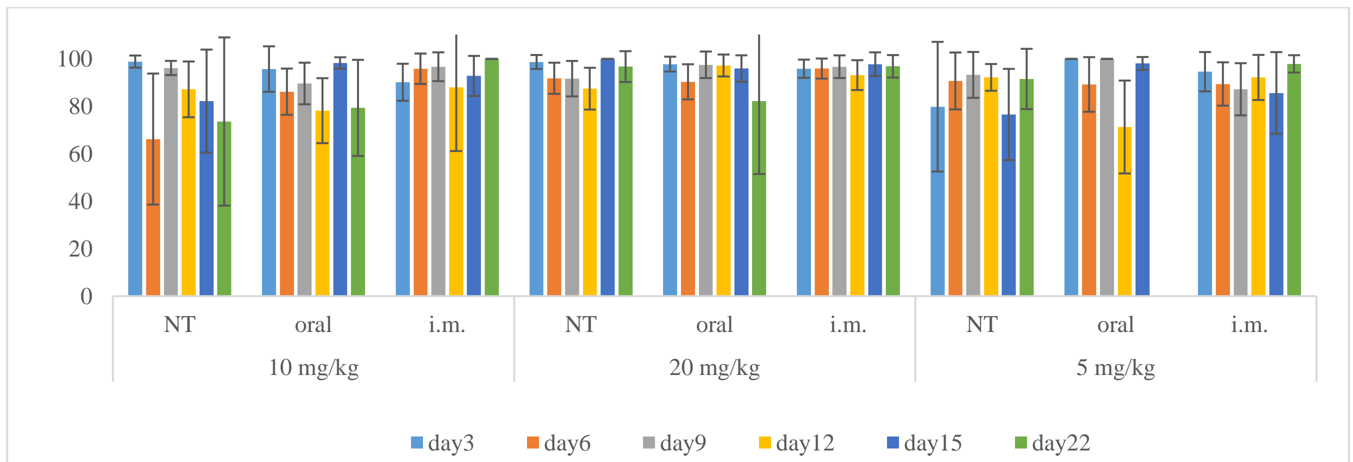

Figure 3. Experiment 2 (inoculation with a bacteriologically fit resistant strain and a bacteriologically fit susceptible strain in a 100:1 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). On Experiment 2, at the last sampling day (Day 22) and for the samples from orally-treated animals (half dose group: 5mg/kg) no E. coli cfu grew on the McConkey plates. NT: No treatment, control group.

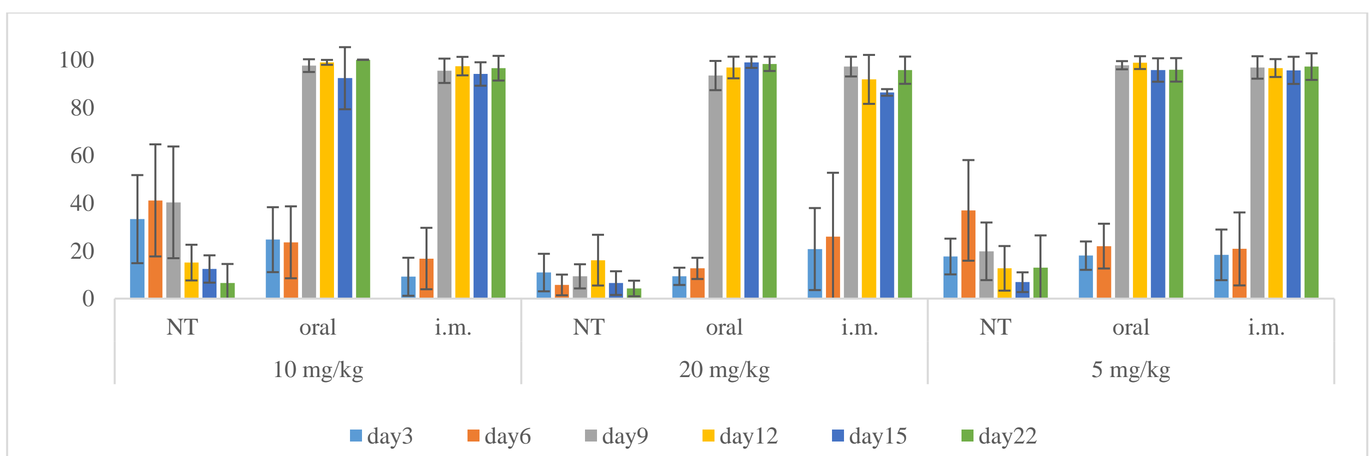

Figure 4. Experiment 3 (inoculation with a bacteriologically fit resistant strain and a bacteriologically fit susceptible strain in a 1:100 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (from day 6 (after sampling) until day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

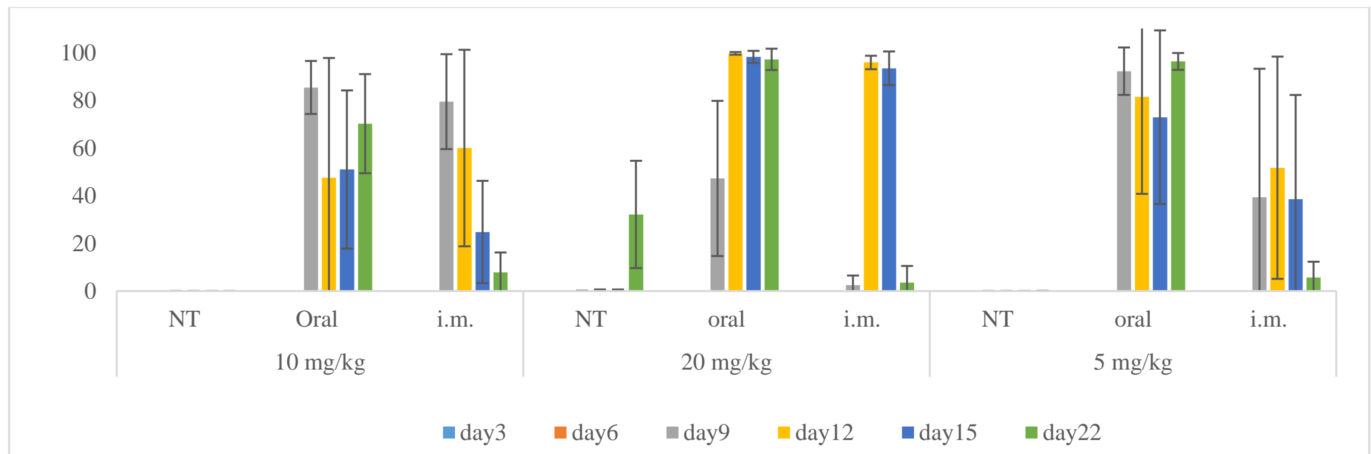

Figure 5. Experiment 4 (inoculation with a bacteriologically non-fit resistant strain and a bacteriologically fit susceptible strain in a 1:100 ratio) results. The y-axis presents the average percentage of resistant strains compared to the total of inoculated strains (retrieved from individual droppings) per group per sampling day. All NT (No treatment, control) groups did not receive enrofloxacin treatment. All treated animals received enrofloxacin treatment for 3 successive days (day 6 to day 8) and the groups are allocated according to the route of administration (oral, i.m.) and treatment dose (proper-, double-, half-dose). NT: No treatment, control group.

A study of the data from the control groups of all four experiments (Figures 2-5) revealed significant differences between the experiments. These differences, which were due to the different inoculums used, were verified by statistical analysis ($p < 0.001$). However, no significant difference ($p=0.304$) was found between the control groups over time, indicating that within each experiment the control groups behaved relatively alike. This indicates that likely no cross-contamination occurred between the treated and the control groups, which suggests that the experimental setup worked.

Effect of strain in the absence of treatment

The results from all non-treated animals (Figures 3-4) show that the animals that were inoculated either with a 100:1 ratio of the fit resistant : fit susceptible strain (Exp. 2) or with the opposite ratio (1:100) (Exp.3) did not show any signs of reversion of antimicrobial resistance towards susceptibility, which suggests that for this strain there appears not to be a fitness cost due to resistance. This finding is in agreement with findings *of in* vitro (Sander et al., 2002) and *in vivo* (Luo et al., 2005) mutational

resistance studies focusing, respectively, on *Mycobacterium* spp. and on *Campylobacter spp.* By contrast, three weeks (Day 22) after inoculation, the non-fit resistant strain could no longer be isolated from the samples taken from non-treated animals that had been inoculated with the non-fit resistant strain either in a 100:1 (Figure 2) or in a 1:100 (Figure 5) ratio.

Effect of treatment

A comparison of the temporal fluctuations of the results from each treated group per experiment (Figures 2-5) shows that there were considerable fluctuations in experiments 1 and 4. This could be attributed to the reduced fitness of the strain that was predominantly inoculated, since the growth potential of bacteriologically non-fit bacteria is difficult to predict *in vivo* (Petersen et al., 2009).

After the treatment period, the control groups from experiments 1, 3 and 4 showed significantly lower proportions of resistant strains compared to the respective treated groups (Table 3). The results of experiments 1 and 4 suggest that when animals are inoculated with non-fit strains, treatment resulted in the persistence of a strain that would normally die out before the end of the experiment (no enrofloxacin-resistant *E. coli* isolates were found on day 22 in all three control groups). The results from experiment 3 suggest that when the inoculum consists of a small proportion of fit resistant strains, the treatment clearly promotes this fit strain, thus allowing it to become the dominant strain. These results are in agreement with the findings of other studies (Jurado et al., 2015; Miranda et al., 2008), where significant differences in resistance rates persisted between the intestinal *E. coli* of the enrofloxacin orally-treated groups and the non-treated groups.

Table 3. Linear mixed models performed per experiment and overall to assess the effect of treatment. Each animal was listed as subject, and sampling as repeated. The repeated covariance type was first-order autoregressive. The dependent variable used was the proportion of the enrofloxacin-resistant colonies to the sum of the resistant and the susceptible colonies.

	Univariate analysis		
Categorical variable	**Estimate**	**Standard Error**	**P-value**
Experiment 1			**.001**
Intercept	55.09	4.56	<.001
No treatment	-45.81	7.96	.001
Treatment (ref)	0	0	.
Experiment 2			**.313**
Intercept	89.07	1.99	<.001
No treatment	-3.45	3.38	.313
Treatment (ref)	0	0	.
Experiment 3			**<.001**
Intercept	96.02	1.20	<.001
No treatment	-82.42	2.09	.004
Treatment (ref)	0	0	.
Experiment 4			**.004**
Intercept	59.73	7.69	.002
No treatment	-56.86	13.31	.004
Treatment (ref)	0	0	.
Overall			**<.001**
Intercept	74.50	3.12	<.001
No treatment	-47.14	5.40	<.001
Treatment (ref)	0	0	.

In contrast, for experiment 2, the findings from the control and the treated groups were similar up to the end of the experiment (p=0.313). The inoculum was largely composed of the fit resistant strain, which apparently colonized the gut of the animals successfully and remained prevalent up to the end of the experiment, irrespective of treatment. Accordingly, Austin *et al.* (1999) suggested that once the resistance prevalence reaches a certain level, antimicrobial use no longer plays a role in the resistance selection. Handel *et al.* (2006) reported that small changes in the volumes of antimicrobials used in a population with a low level of antimicrobial resistance lead to much larger changes in resistance when compared with changes in antimicrobials used at a high level of resistance. Similar effects were seen when comparing antimicrobial use with antimicrobial resistance levels for several classes of antibiotics using data from seven European countries (Chantziaras et al., 2014).

Fitness of the resistant strain and route of administration

All potential effects were tested univariately and the statistically significant effects of strain ($p<0.001$) and administration route ($p=0.044$) were selected and further included in a multivariate linear mixed model. The factors that were finally selected were bacterial fitness of the resistant strain ($p<0.001$) and route of administration ($p=0.052$), as well as the interaction between bacterial fitness and administration route $p<0.001$ (Table 4). Regarding fitness, the inoculation with a fit resistant strain clearly resulted in significantly higher proportions of resistant *E.coli.* When comparing the administration routes, oral administration selected more for resistance. When looking at the interactions, it becomes clear that the combination of oral treatment and a non-fit strain had a significantly larger influence on the outcome. No similar studies have been performed for broiler chickens, but in a somewhat comparable study focusing on pigs and using *Salmonella* enrofloxacin-susceptible and enrofloxacin-resistant strains (Wiuff et al., 2003), selection for resistance among the artificially introduced *Salmonella* was also higher for oral administration than for intramuscular.

Table 4. Linear mixed models performed for all experiments, including data from 120 chickens after they had received enrofloxacin treatment. After assessing univariately the main effects of strain, prevalence of resistance before treatment (inoculum ratio), treatment dose, and administration route, a multivariate model tested the effects of fitness of strain and administration route. In the final mixed linear model, two-way interactions between significant variables were also evaluated (with the significance level set at $P < 0.05$). In all models, to correct for the interdependency of chicks within a pen, pen was included as a random variable.

	Univariate analysis			*Multivariate analysis with interactions*		
Categorical variable	*Estimate*	*Std. Error*	*P-value*	*Estimate*	*Std. Error*	*P-value*
Intercept				92.60	3.97	<.001
Bacterial strain			**<.001**			**<.001**
Intercept	92.69	3.97	<.001			
Non-fit	-35.31	3.08	<.001	-48.10	4.13	<.001
Fit (ref.)	0	0	.	0	0	.
Administration route			**.044**			**.052**
Intercept	67.94	3.26	<.001			
Oral	13.38	4.62	.044	.06	5.63	.992
Parenteral (ref.)	0	0	.	0	0	.
Inoculum ratio			**.223**			
Intercept	77.16	4.18	<.001			
100res:1sens	-5.16	4.22	.223			
1res:100sens (ref)	0	0	.			
Treatment dose			**.764**			
Intercept	72.33	7.44	.002			
10 mg/kg	-0.87	10.50	.994			
20 mg/kg	6.92	10.49	.557			
5 mg/kg	0	0	.			
Interactions strain * administration route						**<.001**
non-fit * oral				25.86	5.88	<.001
Non-fit * parenteral				0	0	.
Fit &* oral				0	0	.
Fit * parenteral				0	0	.

Treatment dose

The treatment dose did not result in significant differences (p = 0.764) with regard to the resistance ratio. This is in agreement with Jurado *et al.* (2015), who found no significant differences among different enrofloxacin dosage schemes that were administered orally to broiler chickens. Although various studies have showed that dosage can have an effect on the emergence of resistance (Olofsson and Cars, 2007; Olofsson et al., 2006; Ozawa and Asai, 2013; Zhao and Drlica, 2001), the results of the present study suggest that it does not have an effect on the selection and spread of resistance. Grouping the referred studies with regards to commensal versus pathogenic *E. coli* isolates, the mutation frequencies of the strains were compared. The mutation frequencies of pathogenic *E. coli* isolates resulted mostly in MPC:MIC ratios of 8 or 16 (Marcusson et al., 2014; Olofsson et al., 2006; Ozawa and Asai, 2013) and were comparable with the mutation frequencies of commensal *E. coli* isolates – including those in this study (ratio of 16). However, it must be noted that the phenomenon of emergence of resistance was not studied in the current *in vivo* experiments. *De novo* resistance mutations could not be assessed in this study design because no fully susceptible inoculum was included.

In accordance with the European Medicines Agency (EMA) guidelines, we used the most commonly recommended dose for chickens and turkeys (10mg enrofloxacin/kg bodyweight per day for 3-5 consecutive days), and, based on this, we calculated the half and the double dose. However, this dose range does not cover the full range of doses available in leaflets across Europe (from 2.5 mg/kg to 20 mg/kg from 2 to 10 days), albeit for different animal species and indications (EMA, 2014). We selected the current dose variation as a starting point for assessing the effect of dosing because deviations from doses below the authorized dosages, together with plasmid mediated

resistance, have been found to enhance resistance (Canton and Morosini, 2011; Couce and Blazquez, 2009; Macia et al., 2011).

Final remarks and further use

The use of isogenic strains allowed for direct comparisons between all *in vivo* experiments since differences between the fit and non-fit strains can be attributed to the point mutations leading to resistance. To our knowledge, this is the first time such *in vivo* experiments have been performed to measure selection for resistance taking into account the prevalence of enrofloxacin resistance in the initial gut microbiota, bacterial fitness of the resistant strain, route of administration and treatment dose. However, the results could have been different if a strain carrying PMQR genes had been used. Although these genes are quite rare in commensal *E. coli* strains isolated from chickens (Abdi-Hachesoo et al., 2017; Oh et al., 2016; Yue et al., 2008), the transfer rate of resistance is expected to be higher in the presence of such plasmids (Robicsek et al., 2006a), yet transfer rate was not evaluated in this study (Chapter 5). Phenomena such as plasmid loss (Sanchez and Martinez, 2012) and plasmid incompatibility (Carattoli, 2013) should also be considered.

The wild-type strain did not show increased MIC levels against enrofloxacin. The enrofloxacin-resistant strain was created *in vitro* via a chromosomal mutation of the parental wild-type strain. *In vivo*, we took all necessary biosecurity measures to prevent the introduction of other strains (via feed, water, air-borne, etc.). Given the fact that the ratio of the resistant strains in the control groups after treating the other groups was not affected (in favour of the resistant strains) in any group and in any experiment, this provided an *in vivo* illustration of the effectiveness of the experimental setting. Thus, the presence of PMQR genes was ruled out.

Overall, the experimental setup made it possible to study and assess several effects concerning the selection of fluoroquinolone resistance. This study provides a basis for selecting and further investigating relevant research topics. By studying the benefits and the limitations of each experiment, one could select the appropriate experimental setting in accordance with the specific research question. For example, one study setup could be selected to focus on treatment effect and its administration patterns (use of non-fit enrofloxacin-resistant strain, oral administration of enrofloxacin and test various treatment schemes), a different setup could focus on selection of antimicrobial resistance (treated animals, use of bacteriologically-fit enrofloxacin-resistant strains and testing inoculums of increasing prevalence), and a third setup could focus on characteristics of reversion of antimicrobial resistance (non-treated animals inoculated with strains of different bacteriological fitness and testing for reversibility of antimicrobial resistance).

Conclusions

For the purposes of this study, a standardized *in vivo* model was developed that can be used to investigate resistance selection in commensal *E. coli* in poultry. Gut colonization with a bacteriologically-fit enrofloxacin-resistant strain and oral administration of enrofloxacin selected more for antimicrobial resistance than colonization with a non-fit resistant strain and parenteral treatment respectively. This novel protocol made it possible to study various factors both selectively and collectively, and to identify the advantages and disadvantages in each case, thus providing insights into treatment strategies using enrofloxacin.

Supplementary material

Table S1. Antimicrobial resistance of *E. coli* IA2 strain (isolated from a faecal sample of a broiler chicken) measured with Etests (Biomerieux, Marcy l'Etoile, France) against several antimicrobials (expressed in mg/L). The epidemiological cut-off values (as set by the European committee on antimicrobial susceptibility testing) were used to determine whether the strain was resistant or not against those antimicrobials.

	Colistin	Trimethoprim	Enrofloxacin	Streptomycin	Nitrofurantoin	Nalidixic acid	Sulphomethoxazole	Chloramphenicol	Ceftazidime	Gentamicin	Tetracycline	Ampicillin	Rifampicin
E. coli IA2 strain	0,094	0,19	0,032	4	6	2	16	4	0,094	1	2	1,5	8
Cut-off values	≤2	≤2	≤0,125	≤16	≤64	≤16	≤64	≤16	≤0,5	≤2	≤8	≤8	-*

*: No cut-off value for rifampicin

Table S2. Primers used in this paper.

Target gene	Primers sequences(5' to 3')	Annealing T (◦C)
DNA gyrase subunit A (*gyrA*)	CGACCTTGCGAGAGAAAT (F) GTTCCATCAGCCCTTCAA (R)	57
DNA gyrase subunit B (*gyrB*)	CTCCTCCCAGACCAAAGACA (F) TCACGACCGATACCACAGCC (R)	60
DNA topoisomerase IV subunit A (*parC*)	TGTATGCGATGTCTGAACTG (F) CTCAATAGCAGCTCGGAATA (R)	55
DNA topoisomerase IV subunit B (*parE*)	TACCGAGCTGTTCCTTGTGG (F) GGCAATGTGCAGACCATCAG (R)	54

Table S3. Treatment dose schemes used.

	Proper dose group (10 mg/kg b.w.[1])	***Half dose group (5 mg/kg b.w.)***	***Double dose group (20 mg/kg b.w.)***
	Parenteral treatment[2]		
Day 6	0.052 mL	0.026 mL	0.104 mL
Day 7	0.06 mL	0.03 mL	0.12 mL
Day 8	0.068 mL	0.034 mL	0.132 mL
	Oral treatment		
Day 6	0.5 mL/L	0.25 mL/L	1 mL/L
Day 7	0.5 mL/L	0.25 mL/L	1 mL/L
Day 8	0.5 mL/L	0.25 mL/L	1 mL/L

[1]: b.w.: bodyweight, [2]: Average weight per chick : 130gr (Day 6), 150gr (Day 7), 170gr (Day 8)

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Chapter 6. Use of a seeder-sentinel *in vivo* animal model to evaluate the effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal *E. coli* in broilers

Use of a seeder-sentinel *in vivo* animal model to evaluate the effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal *E. coli* in broilers

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The effect of a commercial competitive exclusion product on the selection and spread of enrofloxacin resistance in commensal *E. coli* in broilers. *PlosOne (submitted)*

Abstract

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

Methods: A controlled seeder-sentinel animal model with one-day-old chicks was used. In experiment 1, as soon as the chicks were hatched, the animals of two groups were treated with a commercial competitive exclusion product (Aviguard®) and the animals of two groups were left untreated. Three days after hatching, 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, an identical experimental setup was used but additionally all groups except the control 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 by selective plating. Linear mixed models were used to assess the effect of Aviguard® on the selection and spread of fluoroquinolone resistance.

Results: The use of Aviguard® significantly ($p<0.01$) reduced the excretion and spread 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 spread of enrofloxacin resistance when no treatment was administered. Whereas this effect was no longer present when enrofloxacin was administered ($p = 0.70$).

Conclusions: When animals were not treated with enrofloxacin, the treatment of one-day-old broiler chicks with Aviguard® successfully reduced the excretion and spread

of enrofloxacin-resistant *E. coli* irrespectively of bacterial fitness of the resistant strain. However, this beneficial effect disappeared when the animals were treated with enrofloxacin.

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 (Regulation, 2003) and worldwide calls for more prudent use of antimicrobials (Dibner and Richards, 2005; van den Bogaard et al., 2002) . Especially in poultry meat production, high levels of antimicrobial resistance are found due to extensive antimicrobial use (Castanon, 2007). Fluoroquinolones are 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 fluoroquinolone in veterinary medicine is controversial (Landoni and Albarellos, 2015) and increased fluoroquinolone resistance rates from human (*Campylobacter spp.*) and animal (*Campylobacter spp., E. coli)* bacterial isolates have led to restrictions in its use (Belgian Royal Decree of 21/07/2016; Rushton et al., 2014) 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 is associated with a biological fitness cost via the acquisition of mutations (Melnyk et al., 2015) that can negatively affect bacterial metabolism (Gualco et al., 2007; Lindgren et al., 2005; Park et al., 2013). However cost-free mutations (Luo et al., 2005) or

compensatory mutations that ameliorate fitness cost have also been described (Andersson and Hughes, 2010; Marcusson et al., 2009).

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 (Allen et al., 2013; Joerger, 2003). There has been an increasing interest in using non-antibiotic feed additives, including the use of competitive exclusion (CE) products (Ducatelle et al., 2015; Mountzouris et al., 2009). In this study, Aviguard® (Microbial Developments Limited, Malvern, UK),a commercial CE product, was tested on a standardized *in vivo* animal model for its potential effect in preventing the excretion and spread of fluoroquinolone resistance. Aviguard® is comprised by a freeze-dried mixture of live partially-defined commensal bacteria that derived from the gut flora 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 microbial flora and subsequently to prevent pathogens colonizing the gut (Nurmi and Rantala, 1973). The majority of studies has focused on the role of CE in preventing the introduction of pathogenic strains such as in *Salmonella spp.* (Nurmi et al., 1992; Rantala and Nurmi, 1973; Vandeplas et al., 2010), *Campylobacter spp.* (Stern et al., 2001), *E. coli* (Hofacre et al., 2002) and *Clostridium perfrigens* (Abudabos, 2013; Dahiya et al., 2006). 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 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).

Bacterial strains

E. coli strains 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 (Fig 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., 2006). 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 (Tóth et al., 2003).

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 identified and susceptibility testing (E-test®, BioMérieux, Marcy l'Etoile, France) was performed on confirmed (by standard biochemical testing) *E. coli* isolates.

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 gas formaldehyde mixture at the hatchery, but after transportation 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.

The chicks were hatched, they were housed in groups of four or six animals in 1m^2 disinfected plastic boxes in HEPA-filtered stables. All necessary biosecurity measures were taken to avoid any cross-contamination as described before (Chantziaras et al., 2017). Six groups were used in each experiment (groups A, B, D, 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. 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* 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 adititonally 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).

Sampling procedure was identical for both experiments. In total, six faecal samplings took place in each experiment. Starting at day 2 (after hatching), there was a second sampling shortly before the inoculation of the seeder animals on day 5. A third sampling occurred on day 8 (for experiment 2, this was shortly before the start of the enrofloxacin treatment). The remaining sampling days took place on day 11, 18 and 23. 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 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 colony forming units (cfu)/g of faeces was calculated.

Statistical analysis

<u>*Transmission of enrofloxacin-resistant E. coli strains*</u>

For groups A, B, D and E of experiment 1, the basic transmission ratios (R_0) of the bacteriologically non-fit enrofloxacin-resistant *E. coli* challenge strains (IA50) and the bacteriologically fit (IA66) enrofloxacin-resistant *E. coli* challenge strains were estimated using a stochastic infection model. Therefore, we assumed that the process of transmission of *E. coli* IA50 and IA66 among the broilers was in accordance with the susceptible–infectious (S–I) model. Given that the population exists of (S, I) animals,

after an infection occurred, it consists of (S - 1, I + 1) animals. In the model, the number of contact infections, determined by the number of samples that contained enrofloxacin-resistant strains at day 23 after hatching (end of experiment), was the observed variable (Xi). Xi is also called the 'final size' of the outbreak. Because the final size is a discrete stochastic variable, it can only attain whole numbers, and each of these has its own probability. Using the algorithm described by De Jong and Kimman (De Jong and Kimman, 1994), the probability distribution of the final size for the given parameters and start conditions was calulated. Four populations (Groups A, B, D and E of experiment 1) with N = 6 animals, where initially two animals were inoculated with the resistant strains ($I_0 = 2$) and four animals were susceptible ($S_0 = 4$) (non-infected contact-exposed chicks). In the experiment, the probability distribution of the final size was represented by $F(X_i,R_n|N, S_0, I_0)$. We used the maximum likelihood estimator (MLE) to assess the R_n -values. This MLE is calculated numerically from

$$R_0 = \max\prod_{i=1}^{m} F(X_i; R_0 \,|N, S_0, I_0)$$

in which $F(X_i,R_n|N, S_0, I_0)$ is the likelihood function for the observed value X_i, when N, S_0, I_0 are given and m is the number of experiments.

Proportion 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 ≤ 0.05.

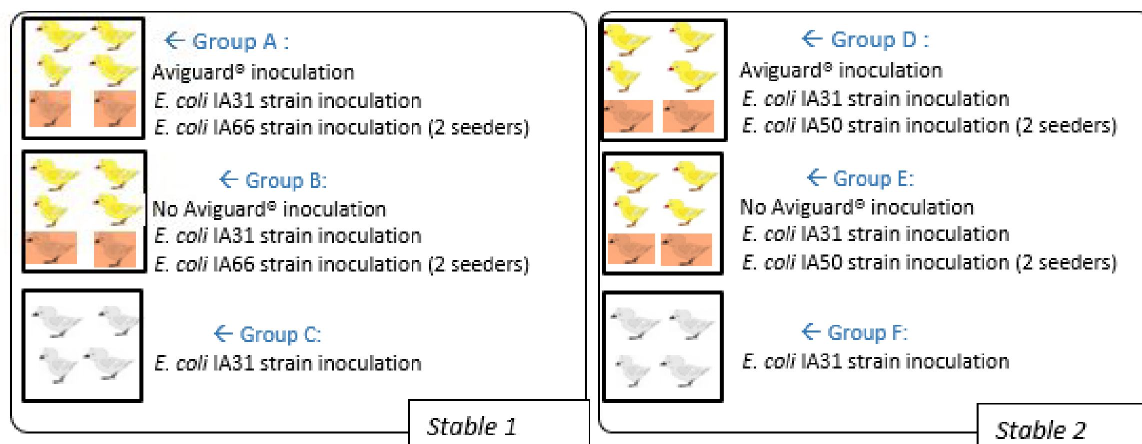
Results and Discussion

Evaluation of the experimental setup

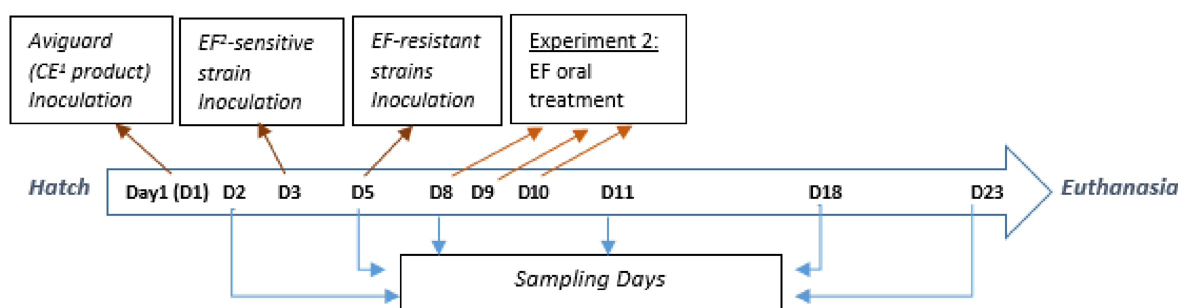
All *E. coli* isolates obtained from plating Aviguard® on McConkey agar were shown to be susceptible for both enrofloxacin and rifampicin and they were not able to grow on enrofloxacin-supplemented agar plates and on rifampicin-supplemented agar plates.

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 (Park et al., 2006; Robicsek et al., 2006). Also, when studying the data from the control groups from both experiments (Fig 4), only colonies

a. Spatial experimental set up



b. Temporal experimental set up



c. Strains used

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

Figure 1. Schematic plan (a. and b.) of the experimental setup for both experiments and strains (c.) used. On Day 1, all animals from Groups A and D were orally inoculated with a competitive exclusion product (Aviguard®). On Day 3, all animals from all groups were orally administered 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 EF-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). On experiment 2, groups A, B, D and E received EF oral 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

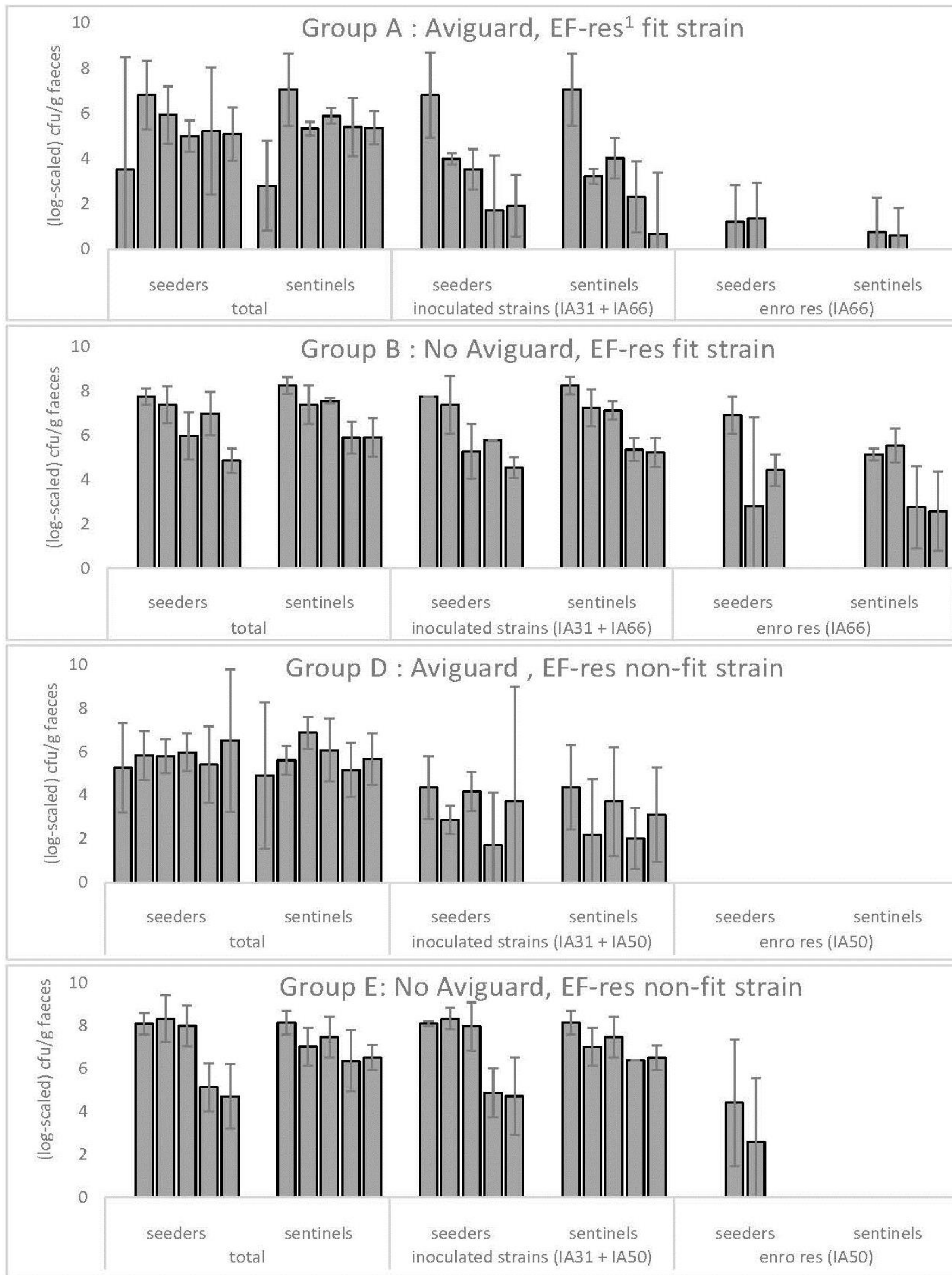


Figure 2. Experiment 1 results. The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). Results on x-axis is presented separately for seeders and sentinels although this distinction 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' indicates the population of enrofloxacin-resistant E. coli.
[1]: enrofloxacin-resistant

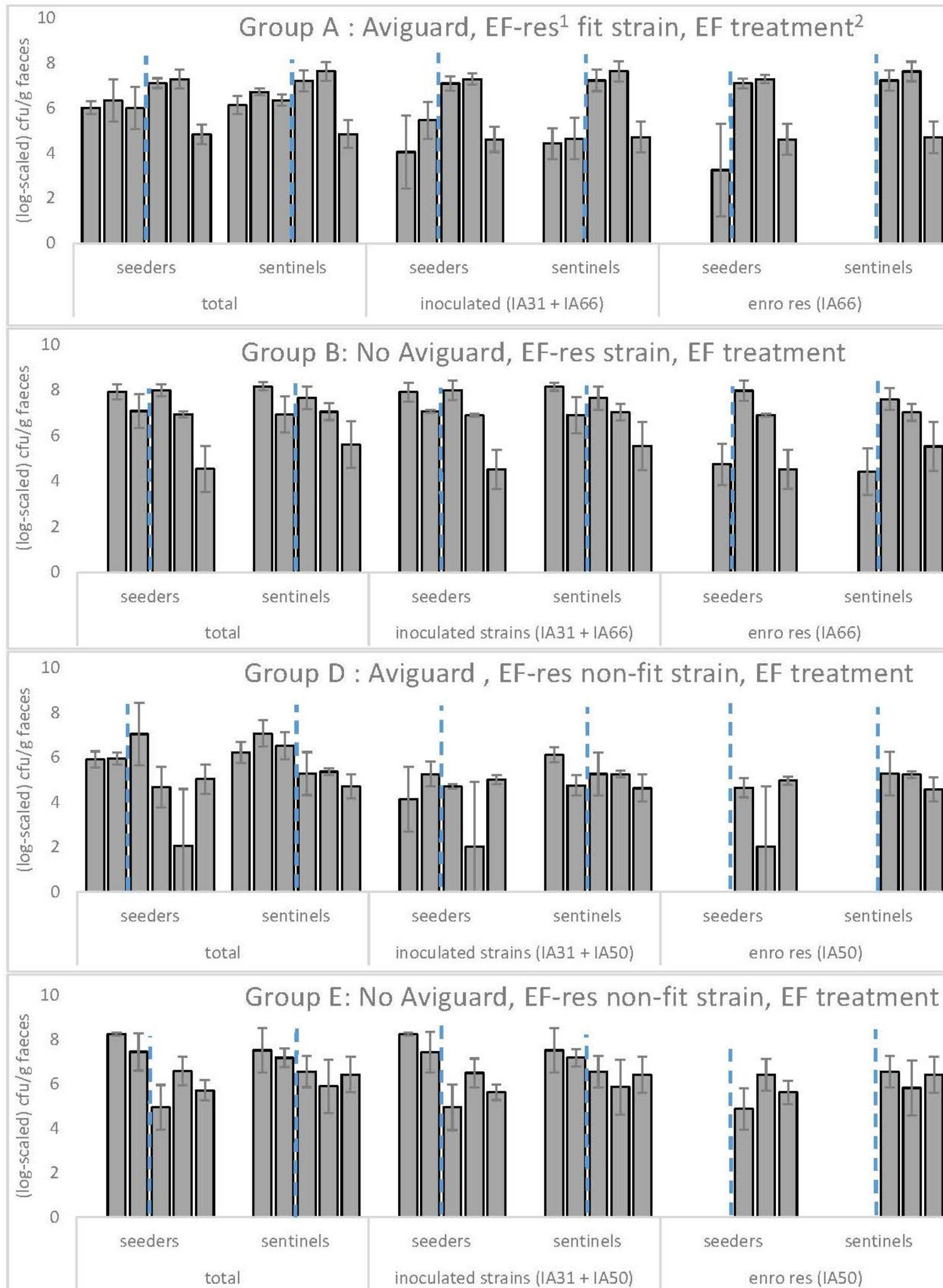


Figure 3. Experiment 2 results. The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 2, 5, 8, 11, 18, 23 as presented on x-axis). Results on x-axis is presented separately for seeders and sentinels although this distinction 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

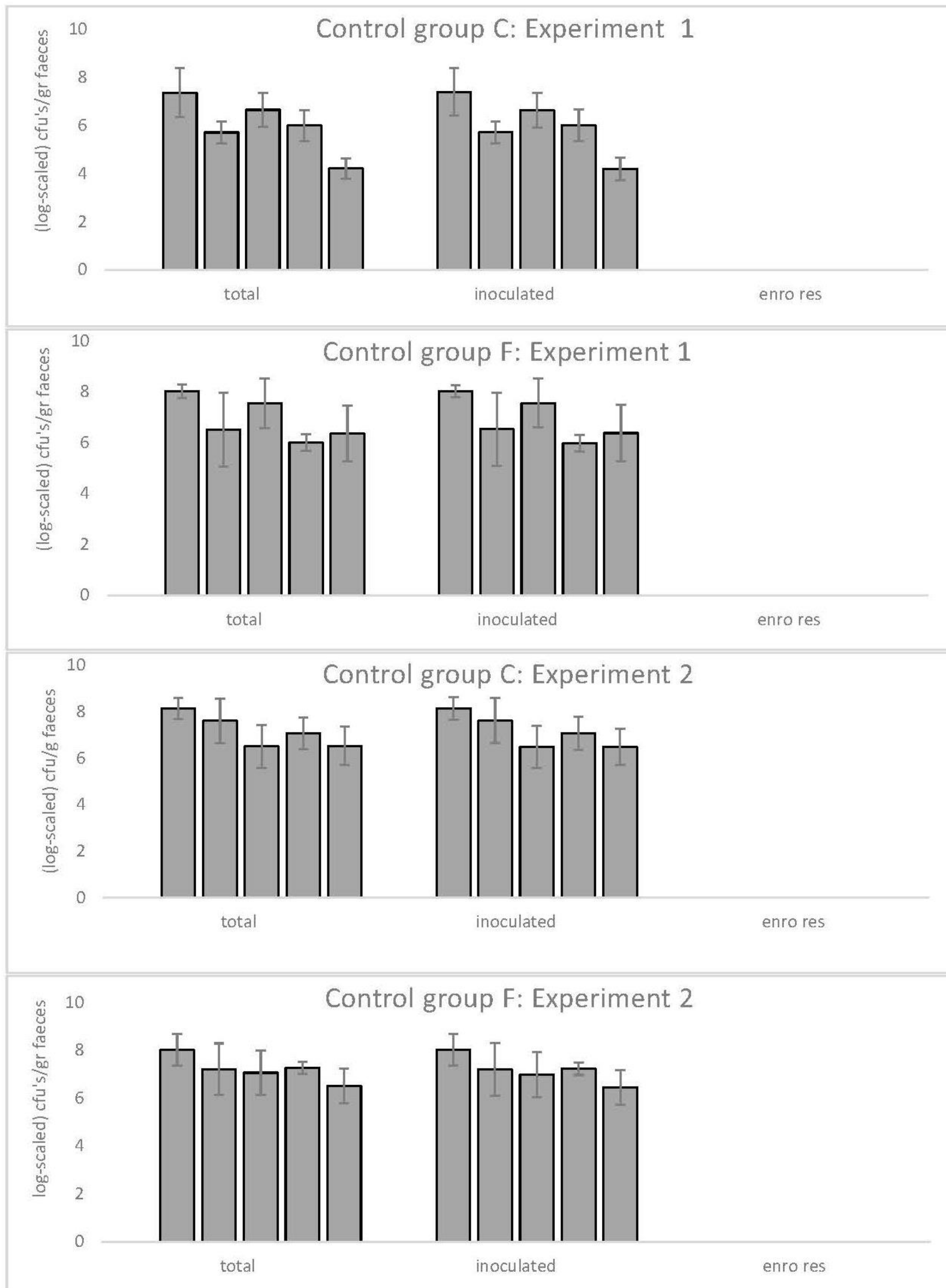


Figure 4. Control groups results for experiments 1 and 2. On Day 3, all animals from all groups were orally administered with a rifampicin-resistant *E. coli* strain (IA31). The y-axis presents the (log-scaled) *E. coli* cfu/g faeces (retrieved from individual droppings) per group per sampling day (Days 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*.

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. 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. 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 prevalence 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; Melnyk et al., 2015; Redgrave et al., 2014), fitness had a significant effect ($p<0.01$) on the transmission of enrofloxacin resistance in the absence of enrofloxacin treatment (Table 1). 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 resistance ($p<0.01$) 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 ($p=0.137$).

In group B (no use of Aviguard®) the R_0 of the fit enrofloxacin-resistant strain (IA66) was positive infinite (+∞) (0.60 - +∞) since the strain managed to spread to all sentinel animals. However, when Aviguard® was previously used (group A), the R_0 of the fit enrofloxacin-resistant strain (IA66) was 0.596 (0.019 – 9.831). For the non-fit enrofloxacin-resistant strain (IA50) and when Aviguard® was previously used (group D), the R_0 could not be calculated as the non-fit enrofloxacin-resistant strain did not colonize any animal (including the inoculated animals). In group E where the non-fit enrofloxacin-resistant strain (IA50) was inoculated but no Aviguard® was administered to the group, the R_0 was 0 (0 – 7,98). Further repetitions of the experiment and a higher group size might have enabled the model to produce more precise estimates and narrower confidence intervals. Nonetheless the current findings clearly suggest that the use of Aviguard® reduces the transmission of both the fit and non-fit strains.

In the absence of treatment (Fig 2), the *E. coli* population originating from Aviguard® was the predominant strain that 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 extended-spectrum cephalosporin (ESC)-resistant *E. coli*, where it was shown that the transmission and excretion of ESC-resistant strain in the absence of antimicrobial treatment was also reduced (Ceccarelli et al., 2017). These results suggest that indeed the use of Aviguard® may have a beneficial effect on the spread of resistance strains. However, while in this work and in the work of Ceccarelli et al. (Ceccarelli et al., 2017) the introduction of the enrofloxacin-resistant strains took place a few days after the administration of Aviguard®, this is not always the case in the field.

Table 1. Linear mixed models performed per experiment to assess the effects of bacterial fitness, Aviguard® and EF-resistant strain transmission (Seeders versus Sentinels).

	Categorical variable / Parameter	*Univariate analysis*			*Multivariate analysis (final model)*		
		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.

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 on β-lactam-resistance (Borjesson et al., 2013; Mo et al., 2016; Mo et al., 2014; Persoons et al., 2011; Projahn et al., 2016), quinolone resistance (Börjesson et al., 2016; Petersen et al., 2006) 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 take place in earlier instances than administrating in one-day-old chicks. This is confirmed by the report

that a CE product administration 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 greatparent and parent stocks, the *in ovo* inoculation of Aviguard® or the spraying of Aviguard® to 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 on 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 become highly prevalent 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 1). 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 cancelling all other effects. To overcome this effect, it has been proposed that Aviguard® could be used after the antimicrobial treatment period to re-establish a susceptible microbial gut flora (Stavric and Komegay,

2008). However, it is questionable if the later inoculated commensal bacterial flora would successfully replace the highly prevalent resistant flora that is expected to be found after antimicrobial treatment as recent findings indicate otherwise (Ceccarelli et al., 2017).

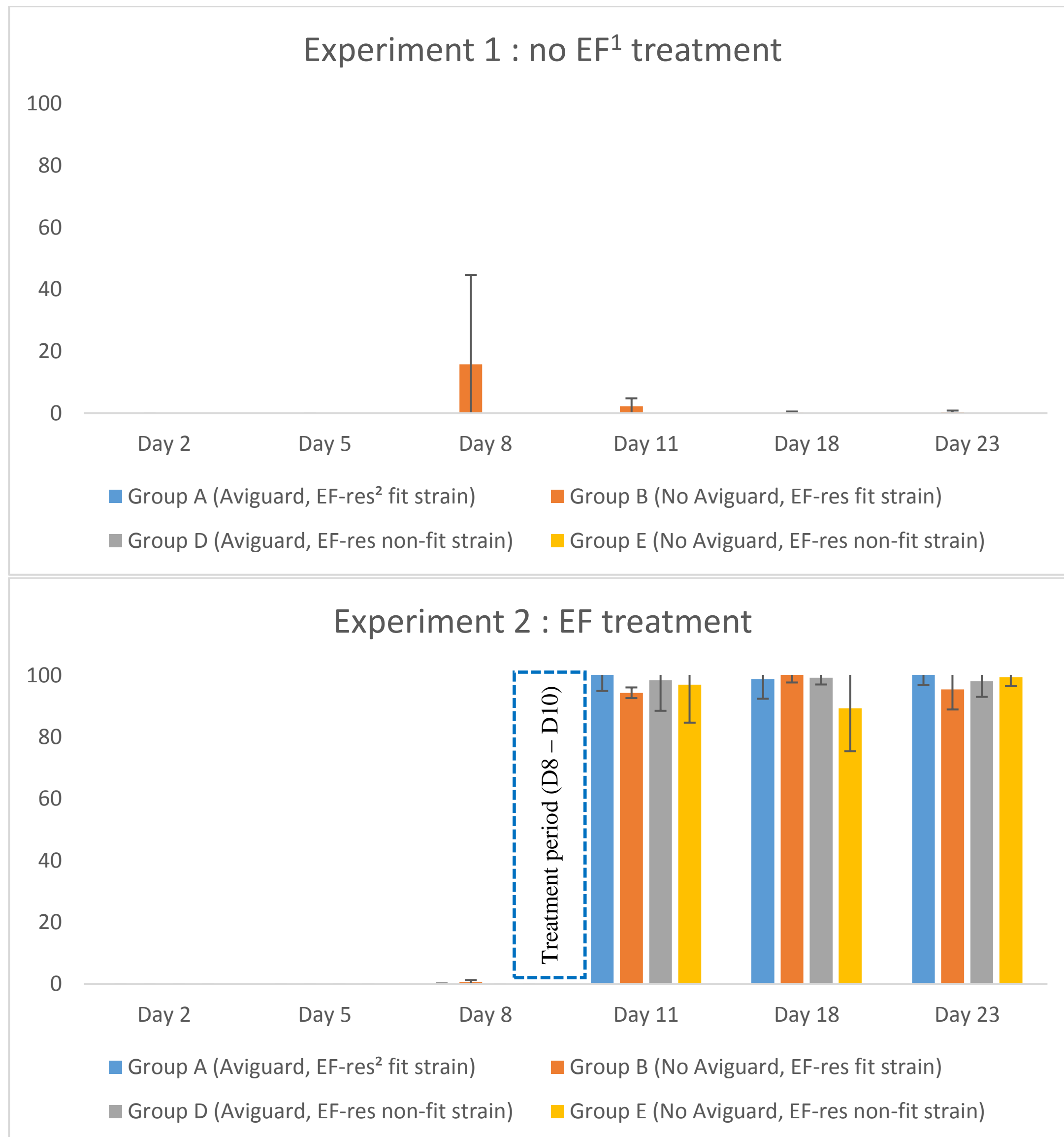


Figure 5. Prevalence of enrofloxacin-resistant *E. coli* strains. On Day 1, all animals from Groups A were orally inoculated with Aviguard®. On Day 3, all animals from all groups were orally administered with a rifampicin-resistant *E. coli* strain (IA31). On Day 5, a bacteriologically-fit strain (IA66) 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

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 cancelling all other effects. Thus, to keep the beneficial effect of this competitive exclusion product, treatment administration should be avoided as much as possible.

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CHAPTER 6

FACULTY OF VETERINARY MEDICINE
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Chapter 7. General Discussion

General Discussion

Epidemiology of antimicrobial resistance: what do we know and what do we miss?

Antimicrobial resistance is an ancient phenomenon (Bhullar et al., 2012; D'Costa et al., 2011; Wright and Poinar, 2012). The emergence of antimicrobial resistance can be seen as a natural defense mechanism employed by bacteria as they are constantly exposed to bioactive molecules produced by bacteria, fungi, plants and many other organisms (Wright, 2007). What is new –closely following the introduction of anthropogenic antimicrobial agents- is the selective pressure that is exerted on both human and veterinary bacterial pathogens and commensals that has lead to the selection and spread of antimicrobial resistance determinants both vertically and horizontally in these bacteria (Schwarz et al., 2017). Once selected, antimicrobial resistance may need a continuous antimicrobial pressure to persist or it may persist even in the absence of any antimicrobial pressure (Andersson and Hughes, 2011). Similarly, reduction of antimicrobial resistance, may occur, especially in the absence of antimicrobial selection pressure (Andersson and Hughes, 2010).

Antimicrobial agents have been used for treating disease, preventing disease and improving feed efficiency in farm animals. Their use was implemented in the 1950s as a way to meet the increasing demand for better feed conversion ratio and higher weight gain (Schwarz et al., 2017). Warnings that antimicrobial resistance found in bacteria from animals may form a threat for public health were promptly raised urging for a prudent use (Swann, 1969), yet the regular introduction of novel antimicrobial agents created a certain degree of over-confidence and optimism to both the scientific community and the public and led to an underestimation of the actual threat (WHO, 2016.). During the last twenty years, the alarmingly increasing resistance prevalences

to antimicrobial agents have urged both science and the public to respond by acquiring knowledge on the origins and mechanisms of antimicrobial resistance and by monitoring antimicrobial use and antimicrobial resistance prevalence in pathogenic and commensal bacteria (Canton and Morosini, 2011). However, there is still a lack of knowledge on the *in vivo* epidemiology of antimicrobial resistance and the factors contributing to the selection and spread of antimicrobial resistance at animal and population level (Boerlin and White, 2013; Holmes et al., 2016). Accordingly, this work aimed at contributing to the insight in the epidemiology of antimicrobial resistance by conducting epidemiological studies and employing controlled *in vivo* models that were used to investigate the fluoroquinolone resistance selection and spread in poultry.

Using national surveillance systems to understand epidemiological links between antimicrobial use and resistance and to evaluate intervention efficiency.

Harmonising data is a prerequisite

Throughout the last two decades, several EU member states have made great progress towards monitoring antimicrobial resistance in farm animals (Bager, 2000; Martel et al., 2000; Moreno et al., 2000; NORM-VET, 2016; Schwarz et al., 2007; SVARM, 2016). However, only in recent years, after clear calls for the need of harmonization of resistance monitoring programs (Marion, 2012; Schwarz et al., 2010; Silley et al., 2011; Silley et al., 2012; Wegener, 2003; White et al., 2001), are national reports being published that use more or less uniform methodology on antimicrobial susceptibility testing, allowing for better comparison between countries (EFSA, 2012). Another step forward was the implementation of epidemiological cut-off values, set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Moreover,

the European Food Safety Agency (EFSA) has since 2013 stipulated that reports on the occurrence of antimicrobial resistance in the indicator *E. coli* should become mandatory and be included in each national surveillance program of an EU member state (EFSA, 2012).

Regarding the knowledge of veterinary antimicrobial use in Europe, huge improvement has been made through the activities of European Medicine Agency (EMA). EMA has set up in April 2010 the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project following a request from the European Commission for the Agency to develop a harmonised approach for the collection and reporting of data on the use of antimicrobial agents in animals from EU and European Economic Area (EEA) Member States.

When correlating antimicrobial usage data to antimicrobial resistance data collected from seven European countries (Chapter 3), surprisingy high correlations for all antimicrobial agents tested were observed. Countries with a high antimicrobial use were consistently showing the highest percentages of antmicrobial resistance prevalence to the antimicrobial agents tested. This finding highlights the need for policies promoting lesser and more controlled use of antimicrobials, especially in countries with high levels of antimicrobial use. Apart from that, the need for more data, further detail in data collection and further harmonisation of the data was adressed as well. More specifically, data from more countries should provide a more accurate depiction of the situation in the EU. More detailed (*e.g.* antimicrobial usage data per animal species, farm-level data) and more harmonized data could allow scientists to identify differences in national policies on controlling antimicrobial use, veterinarians' prescribing and dosing habits per country and pharmaceutical marketing strategies. Furthermore, such data would allow to properly study the importance of animal

demographics and to adress specific needs for antimicrobial use in specific countries related to specific diseases (Chantziaras et al., 2014a).

When comparing the national studies using commensal bacteria with pathogenic bacteria again the same need for harmonisation of the data applies to allow for valid comparisons (Chapter 4). Nevertheless, it could be concluded that pathogenic porcine *E. coli* strains were significantly more resistant to four out of ten tested antimicrobials compared to the commensal porcine *E. coli* isolates. In addition, bovine pathogenic strains were significantly more resistant for eleven out of twelve antimicrobials compared with bovine commensal *E. coli* strains. It therefore seems that bovine pathogenenic *E. coli* strains are more multi-resistant than porcine pathogenic *E. coli* strains. In Belgium, prophylactic group treatment with antimicrobials has been largely applied in pigs (98% of pig farms in 2010) (Callens et al., 2012) and has been used more than in bovines (Filippitzi et al., 2014; Pardon et al., 2012). As a consequence, healthy pigs likely have been exposed more to antimicrobial agents than healthy bovines which is translated in higher levels of resistance in the commensal flora. Likewise, porcine commensal *E. coli* strains were more multi-resistant than the bovine commensal *E. coli* strains again an illustration of the fact that antimicrobial agents select for antimicrobial resistance. In general, when comparing pathogenic with commensal bacterial strains, the increased prevalence of multi-resistance for pathogenic strains is best explained by the higher selection pressure exerted on pathogens by repeated treatments (Boerlin et al., 2005; Boerlin and White, 2013; Martinez and Baquero, 2002). Apart from that, the use of antimicrobial agents has well been linked with the transfer of mobile genetic elements conferring virulence determinants (Chapter 3) and hence can increase the virulence of the normal bacterial flora. The propagation of these virulence factors within bacterial communities could

lead to the emergence of new virulent strains from the commensal microflora of animals posing additional public health threats (Boerlin and White, 2013). These findings stress once more the need for prudent use of antimicrobials in the field.

Current situation and future perspectives

On national level, Belgian initiatives have led to the decrease of antimicrobial use in animals during the last years (BelVetSAC, 2014, 2015), and in turn led to decreasing temporal trends of antimicrobial resistance to the majority of the used antimicrobial agents (Hanon et al., 2015). This finding coresponds with the resistance trends from other European countries with similar initiatives *e.g.* the Netherlands (Central Veterinary Institute of Wageningen University, 2015) and Denmark (Danish National Veterinary Institute, 2016).

On European level, the latest reports on antimicrobial resistance data (EFSA and ECDC joint scientific report (ECDC, 2017)) and antimicrobial use data (Sixth ESVAC report (EMA, 2017a)) in the European Union include not only data from more countries but more detailed and further harmonized data compared to previous reports. In 2016, the sixth ESVAC report was published presenting the results on antimicrobial consumption in animals in 2014 in twenty nine European countries (EMA, 2016) –ten countries more compared with the 2012 report (EMA, 2012). By having yearly data, the report revealed important information on the overall declining trends of the sales of antimicrobial agents per country and the percentage of sales attributed to each antimicrobial class. However, the report still could not inform of usage data per animal species. EMA has recently published the draft guidance for reporting antimicrobial consumption data by animal species for public consultation to help all involved countries overcome this limitation (EMA, 2017b). Currently, only Denmark and the Netherlands report detailed per species antimicrobial consumption data. This is

possible since all antimicrobial prescription data are electronically monitored. In Belgium, the data collection at herd level is implemented in the pig production since 2014 (AB –register), and the new Royal Decree of 21/07/2016 in Belgium on the terms of use of drugs by veterinarians and animal management in Belgium (AFSCA/FAVV, 2016) has created the legal basis to enlarge this to the major food producing animal species through the setup of a central electronic database (namely SANITEL-MED) to register all antimicrobial use prescribed for food-producing animals in Belgium per species. These data will in the future allow to better evaluate the effects of interventions. For instance, in Denmark, detailed per animal species data on antimicrobial use and on resistance allowed to estimate the effect of various national interventions. The Danish Monitoring Program (DANMAP) has helped to document the effect of a wide-scale five-year national intervention (*i.e.* the veterinary advisory service contracts of cattle and pigs, mandatory since 2010), or allowed for even more specific interventions such as the assessment of a new legislation concerning treatment of groups of pigs for the year 2015 (Danish National Veterinary Institute, 2016).

Overall, surveillance systems are a prerequisite for understanding the epidemiology of antimicrobial resistance, providing relevant risk assessment data, and evaluating targeted interventions. Resistance monitoring should also allow for the identification of emerging or specific patterns of resistance (ECDC, 2017). Finally, with the use of novel statistical approaches (e.g. Big Data analysis), more detailed data will subsequently lead to more detailed and specific initiatives for both the public and the farming industry.

Epidemiology of antimicrobial resistance: (where) can we intervene?

Although stricter national regulations regarding antimicrobial use and their disposal are gradually taking place, it is hard to imagine a total ban of antimicrobial agents in the veterinary medicine (Van Boeckel et al., 2015). On a global scale and after the various initiatives to lower the use of antimicrobials in food animals, it is still twice as high as the use in humans (Aarestrup, 2012). Antimicrobials are used to control bacterial infections of the animals and are still indisplacable in veterinary medicine (Schwarz and Chaslus-Dancla, 2001). Excepting the obvious –and non-avoidable- effect of the use of an antimicrobial agent towards the selection of resistance to that antimicrobial agent, factors either or not relating to the use of antimicrobials can also play a role, as reviewed in the introduction of this thesis (Chapter 1). Only by thoroughly studying such factors one could identify critical points to intervene and reduce the selection and spread of antimicrobial resistance. To be able to get fundamental insight in the role of such factors, standardised *in vivo* animal models were developed and used. In the first experimental setup, the effects of the treatment dose and the administration route on the selection and spread of resistance was evaluated. Moreover, this experimental setup allowed to study the effect of the initial prevalence of enrofloxacin resistance in the *E. coli* gut microbiota and the effect of the bacterial fitness of the enrofloxacin-resistant strain. The second experimental setup was developed and used to assess the intervening effect of a competitive exclusion product (Aviguard®) on decreasing the spread of fluoroquinolone resistance in poultry in the absence or presence of fluoroquinolone treatment.

Overall, the effect of administering enrofloxacin was non-surprisingly the most impactful effect leading to the selection (Chapters 5, 6) and spread (Chapter 6) of enrofloxacin-resistant strains in the broiler gut *E. coli* flora. This finding is in agreement with other recent experimental *in vivo* studies that tested the impact of administration of enrofloxacin (Jurado et al., 2015) or ceftiofur (Baron et al., 2016) on the selection of antimicrobial resistance in broiler chickens. In addition, the development of current standardised animal models allowed for simultaneous evaluation of various other effects for their role on the selection of fluoroquinolone resistance in commensal *E. coli* in broilers, such as route of administration of the treatment and treatment dose.

Focusing on administration route, oral treatment exerted a higher selection pressure in commensal *E.coli* in broilers compared to parenteral enrofloxacin treatment (Chapter 5). This finding is in agreement with the findings of other *in vivo* studies that were performed on pigs or on mice (Wiuff et al., 2003; Zhang et al., 2013). In Chapter 5, it was seen that shortly after the parenteral administration of enrofloxacin was stopped, a gradual reduction of resistance prevalence was seen for the non-fit enrofloxacin-resistant *E. coli* strain while after oral administration of enrofloxacin, the resistant strain remained prevalent until the end of the experiment. This effect was mainly seen when a non-fit enrofloxacin-resistant strain was used. In literature, it has been shown that fitness loss can be reflected in a reduced growth rate (Andersson, 2006; Majcherczyk et al., 2008), a reduced transmission rate (Randall et al., 2008), a higher clearance rate (Gustafsson et al., 2003) and a decreased invasiveness (Fernebro et al., 2008), which can make the resistant strains less competitive than fit susceptible strains in the absence of antimicrobial selection pressure. Indeed, when co-inoculated both *in vitro*

and *in vivo,* the susceptible population quickly outcompeted the bacteriollogically non-fit enrofloxacin-resistant population (Chapter 5 and preliminary *in vivo* studies, data not shown). Thus, in the field where there is a plethora of bacteria possibly showing different levels of fitness, the intensity of selection could be reduced by using parenteral routes of treatment (*i.m., i.v.)* compared with oral treatment. Moreover parenteral administration routes generally are administerd to individual animals and therefore are lesser frequenty used for group treatments. On the other hand, it was shown (Experiment 3 of Chapter 4) that once a fit resistant strain is present in high ratio, it is expected to remain highly prevalent irrespectively of treatment. Colonization with fit resistant strains at a very young age, as used in the current experimental models, might contribute to obtaining such a stable resistant flora.

With respect to treatment dose, no significant effect of dose in the selection of fluoroquinolone resistance was observed (Chapter 5). This might seem surprising given the literature referring to the mutant prevention concentration (MPC) concept and the effect of fluoroquinolone dose (Drlica et al., 2012; Olofsson and Cars, 2007; Olofsson et al., 2006; Zhao and Drlica, 2001). Yet, while the experiments from this thesis focus on the selection and spread of existing fluoroquinolone resistance, the MPC concept focuses on the selection of emerging (fluoroquinolone) resistance (Marcusson et al., 2014; Olofsson and Cars, 2007; Olofsson et al., 2007). Our experiments show that for any dose that was used, there was an equal fluoroquinolone resistance selection pressure (Chapter 5). The aforementioned *in vivo* results prove that the MPC concept –albeit useful- should not be generalised to the whole epidemiology of fluoroquinolone resistance (Courvalin, 2008).

Besides mutational-induced resistance, horizontal transfer of resistance determinants plays an important role for the spread of fluoroquinolone resistance (Binnewies et al.,

2006). Horizontal gene transfer via plasmids has been increasingly studied as this seems to be the most common 'vehicle' of introducing resistant determinants to bacteria (Carattoli, 2013). Moreover, plasmid's ability to transfer resistance genes from ecologically and taxonomically distant bacteria is of special importance for public health (Carattoli, 2009; Liu et al., 2016).The potential role of plasmid incompatibility as a way to prevent the *in vivo* insertion of horizontally-transmitted resistance genes was highlighted in a preliminary *in vivo* experiment (data not shown). Future research using strains with different resistant mechanisms, will provide more insights in the role of these mechanisms in the epidemiology of resistance.

Using in vivo experimental studies to evaluate possibilities to decrease the colonisation and spread of antimicrobial resistance

Feed additives (competitive exclusion products, probiotics, prebiotics), bacteriocins, bacteriophages or vaccinations all have attracted attention as potential means to decrease the use of antimicrobial agents in veterinary medicine (Allen et al., 2013; Caly et al., 2015; Huyghebaert et al., 2011; Joerger, 2003; Pedroso et al., 2014). These agents have been studied *in vivo* for their role in disease prevention for various bacterial pathogens (Abudabos, 2013; Dahiya et al., 2006; Hofacre et al., 1998; Mountzouris et al., 2009; Tellez et al., 2012), the reduction of subclinical infections (Brennan et al., 2003), the immune response of animals (Kabir et al., 2004; Kabir, 2009) or generally on production efficiency and quality (O'dea et al., 2006; Stavric and Komegay, 2008). For the needs of this thesis we focused on the competitive exclusion (CE) concept and its potential effect on the colonisation and spread of antimicrobial resistant genes. The reason for focusing on the use of a CE product was the consequence of results obtained from the *in vivo* experiments (Chapter 5). There it was seen that early colonization with a fit resistant strain resulted in a stable highly

prevalent resistant flora (irrespectively of treatment). As a consequence it was hypothesized that introducing a stable susceptible flora prior to the introduction of any resistant strains, might prevent the colonisation by resistant bacteria. The CE concept has first been used to control a *Salmonella infantis* outbreak in poultry in 1971 (Nurmi et al., 1992) and since then an extensive bibliography relating to the use of competitive exclusion products has been produced. Including a limited amount of *in vivo* research on the 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, focusing either on pathogenic *E. coli* (Hofacre et al., 2002) or on extended-spectrum β-lactamase producing *E. coli* (Ceccarelli et al., 2017; Nuotio et al., 2013).

In this thesis, two *in vivo* experiments were conducted to quantify the effect of a commercially available CE product (Aviguard®) on the colonisation and spread of fluoroquinolone resistance in commensal *E.coli* in broilers, using well-defined and controlled experimental *in vivo* models and taking into account the effect of enrofloxacin treatment. In the absence of treatment (Chapter 6), the *E. coli* population originating from Aviguard was the predominant strain that largely prevented the establishment and spread of enrofloxacin-resistant *E. coli* strains that were introduced afterwards. This is in agreement with the results of a recent study focusing on the effect of the use of Aviguard® on extended-spectrum cephalosporin (ESC)-resistant *E. coli*, where it was shown that the transmission and excretion of ESC-resistant strain in the absence of antimicrobial treatment was also reduced (Ceccarelli et al., 2017). This experiment showed that the early establishment of a stable susceptible flora indeed can form a barrier for resistant strains at later timepoints. However, when enrofloxacin was administered to the animals, enrofloxacin-resistant strains quickly overgrew the susceptible flora and was spread within the groups very effectively. Thus, to keep the

beneficial effect of this competitive exclusion product, treatment administration should be avoided as much as possible. The effect of reinoculation of such CE products to treated animals shortly after stopping the antimicrobial treatment could be studied. One important point of attention is however that with respect to the antimicrobial resistance problem, there is also a clear need to monitor CE products for the potential presence of antibiotic resistance genes as well (D'Aimmo et al., 2007).

The role of the veterinarian to combat antimicrobial resistance

Another way to combat antimicrobial resistance is to minimize the empirical use of antimicrobials. Proper education and transdisciplinary exhange of information between medical and veterinary personnel who prescribe antimicrobials could lead to a better understanding of the societal burden of suboptimal antimicrobial use (Goff et al., 2017; Goldstein et al., 2016). Moreover, restriction of group treatments and promotion of individual treatment protocols are expected to reduce the intensity of antimicrobial selection pressure in the field (Chapter 5). Furthermore, the availability of faster diagnostic tools is expected to enable the veterinarian or the medical doctor to avoid treatment delays or unnecessary use of antimicrobials (Bauer et al., 2014; Caliendo et al., 2013; Dumoulin et al., 2017).

Epidemiology of antimicrobial resistance: Insights for the broiler industry

The broiler industry is by far the most industrialized animal sector (Flanders and Gillespie, 2015). Growth performance and viability of broilers have been positively effected by the use of antimicrobials (da Costa et al., 2011). On the other hand, high resistance rates against the most critically important antimicrobial classes for veterinary and human animal health have been observed in broiler chickens in several European countries (Chantziaras et al., 2014a; Chantziaras et al., 2014b; Dorado-García et al., 2016; Kmeť and Kmeťová, 2010). Moreover, the transfer of resistant

determinants between broilers and humans has been demonstrated (Dierikx et al., 2013; Jakobsen et al., 2010). Apart from that, highly sanitized industrial housing facilities and lack of contact with breeder chickens can slow down the intestinal maturation resulting in an less diverse and stable microflora and possibly an imbalance between protective and harmful bacteria (Chan et al., 2013; Ducatelle et al., 2015). Regarding the current situation on antimicrobial use in broilers, early antimicrobial treatment in the first days after hatching is still common practice. Thus, the immature and probably simple commensal bacterial flora that is expected to be found in these chicks is wiped out in its earliest stage of development. Since the bacterial flora of these chicks has very little time to recover from this (they are slaughtered 5 - 7 weeks later) and do not have access to many sources of different (intestinal) bacteria, this selection for resistant bacteria at very early age might extend until the age of slaughter, especially when the selected resistant strains are fit (Chapters 5 - 6). It is very important hence to try to maximize the time between hatching and the first treatment and additionally to try to get the intestinal flora to mature as quickly as possible so that a stable, diverse microbiota is achieved before any antimicrobial treatment. This might help the microbiota to recover (outselect the resistant strains) faster and preferably before the age of slaughter.

Since the ban of antimicrobial growth promoters, a poorly described disease syndrome (referred to as 'dysbacteriosis') is becoming more prevalent (Huyghebaert et al., 2011) complicating the efforts for minimizing antimicrobial use. Lack of contact between broiler chickens with the breeder chickens have slowed the development of mature ceacal microflora resulting in an imbalance between protective and harmful bacteria (Chan et al., 2013; Ducatelle et al., 2015). As a response, there has been an increasing interest in using non-antibiotic feed additives such as use of competitive exclusion

products among others (Ducatelle et al., 2015; Mountzouris et al., 2009). Apart from that, the importance of biosecurity has been increasingly stressed in various recent publications (Chan et al.,2013; Ducatelle et al., 2015).

CE concept and the poultry production pyramid : where to intervene?

CE in poultry refers to the administration of commensal adult intestinal microorganisms (undefined or defined probiotic cultures) in newly hatched chicks to quickly induce the formation of a diverse yet stable intestinal microbial flora and subsequently prevent pathogens colonizing the gut (Nurmi and Rantala, 1973). 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 on β-lactam-resistance (Borjesson et al., 2013; Mo et al., 2016; Mo et al., 2014; Persoons et al., 2011; Projahn et al., 2016), quinolone resistance (Börjesson et al., 2016; Petersen et al., 2006) 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®) could be even more beneficial when used in earlier instances than administrating in one-day-old chicks in broiler farms. This is confirmed by the report that a CE product administration 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 the use of Aviguard® in greatparent and parent stocks, the *in ovo* inoculation of Aviguard® or the spraying of Aviguard® to embryonated eggs, before exposure to antimicrobial treatments or resistant strains, should be studied as these applications could potentially help more to reduce the prevalence of antimicrobial resistant determinants.

Improve biosecurity to combat antimicrobial resistance?

Biosecurity measures have been shown to be of high importance for animal health (disease prevention) (Gelaude et al., 2014; Postma et al., 2016) and food safety (Collins and Wall, 2004; Humphrey, 2004). The results of this thesis also showed the benefits of applying strict biosecurity measures. In both *in vivo* experimental studies, all necessary biosecurity measures were taken to avoid any cross-contamination (feed, water, indirect contact, air-borne transmission) between groups. Also, detailed cleaning and additional disinfection steps were applied to ensure the high biosecurity level of the studies. As a result, the animals of the control groups (Chapter 6) that were administered only with an enrofloxacin-susceptible *E. coli* strain and were not treated with enrofloxacin only carried enrofloxacin susceptible strains until the end of both performed experiments. Although the biosecurity levels differ in the field, and resistant determinants are expected to be more prevalent, these results show that biosecurity measures can help in avoiding the spread of resistance, for example between farms or within a farm. Indications for the latter were seen in pigs when using field data (Laanen et al., 2013).

Final conclusions

The main focus throughout this thesis was on the epidemiology of antimicrobial resistance. The main finding was that the use of antimicrobials has a profound effect on the selection and spread of antimicrobial resistance. This was confirmed by investigation of both national and international data on the use of antimicrobials and the presence of antimicrobial resistance and by using the standardised *in vivo* experimental animal models. Standardized *in vivo* models enabled us to study selectively and collectively various treatment strategies on the selection and spread of fluoroquinolone resistance. More specifically, it was shown that parenteral

administration is preferred as it exerts overall a lower selection pressure than oral administration. Finally, the use of a CE product was able to reduce colonization and spread of enrofloxacin resistance but only in the absence of treatment.

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CHAPTER 8.

SUMMARY

Chapter 8. Summary

Resistance to antimicrobial agents is one of the biggest threats to global health, food security, and economic development today. A wide recognition of this global concern by science and the public has led to an increasing number of studies acquiring knowledge on the origins and mechanisms of antimicrobial resistance and also led to the establishment of several national surveillance programs to measure antimicrobial usage for human and veterinary medicine and to monitor the antimicrobial resistance levels in commensal bacteria in humans and in food producing animals. However, there is still a lack of knowledge on the epidemiology of antimicrobial resistance and the factors contributing to the spread and selection of antimicrobial resistance at animal and population level.

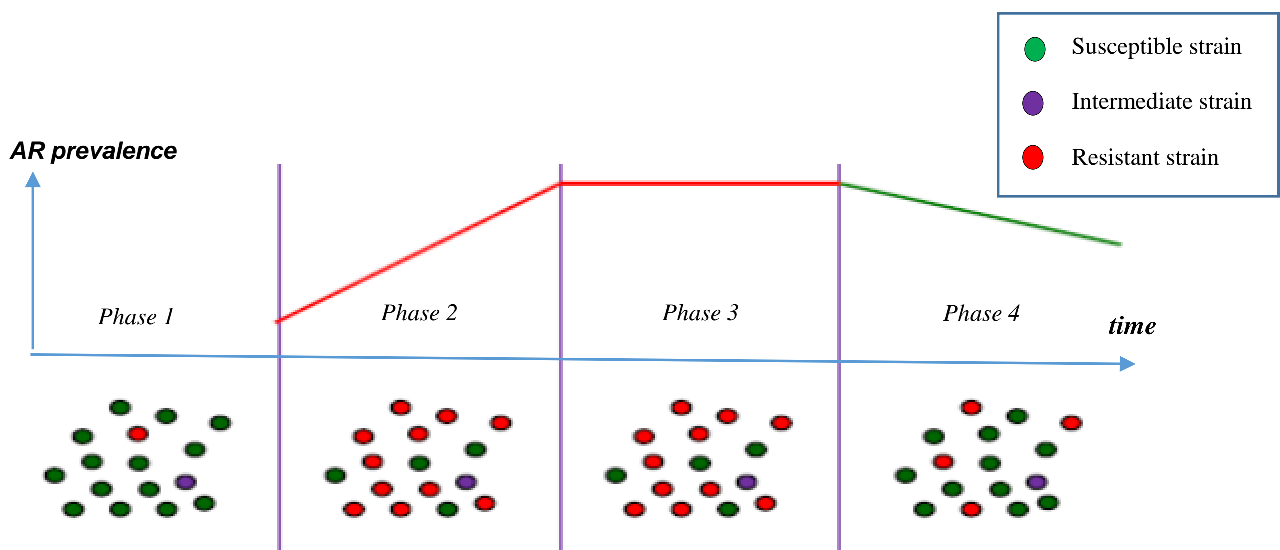


Figure 1. Epidemiology of antimicrobial resistance (AR). Four phases are described: Phase 1. Emergence of AR, Phase 2. Selection and spread of AR, Phase 3. Persistence of AR, Phase 4. Reduction of AR. During Phase 1 the majority of the strains are susceptible. Under selection pressure (e.g. use of antimicrobials) the susceptible strains decrease and the resistant strains become the majority (Phase 2) and persist (Phase 3). In the absence of selection pressure, a reduction of AR prevalence may occur (Phase 4).

The epidemiology of antimicrobial resistance, albeit complex, can be rougly presented as a succession of four phases (Fig. 1). The general introduction of **Chapter 1.1.** reviews key aspects of selection, spread and persistence of AR (phases 2 and 3) through antimicrobial use in farm animals. However, besides the use of antimicrobials, other factors can be involved in the selection and spread of antimicrobial resistance determinants. Thus, a detailed overview of all important non-antimicrobial factors that may influence the selection and spread of antimicrobial resistance is given in **Chapter 1.2.**.

Based on data from publicly available national and international monitoring reports of seven European countries, correlations between antimicrobial use in food-producing animals and resistance prevalence for commensal *Escherichia coli* isolates originating from pigs, poultry and cattle, for eight antimicrobial agents were evaluated in **Chapter 3**. The link between the quantity of antimicrobials administered to food producing animals per country (expressed in mg/population correction unit) and the prevalence of antimicrobial resistance (interpreted by EUCAST epidemiological cut-off values) in *E. coli* isolates (4831 isolates in total) to antimicrobial agents representing the different antimicrobial classes used, was studied. For all antimicrobial classes studied remarkably high correlation coefficients were obtained, indicating that, at a national level, the level of use of specific antimicrobials strongly correlates to the level of resistance towards these agents in commensal *E. coli* isolates in pigs, poultry and cattle. However, data restraints reveal the need for further detail in collection and harmonization of data concerning antimicrobial use and antimicrobial resistance data in Europe.

In **Chapter 4**, detailed studies on antimicrobial resistance for commensal *E. coli* (in broilers, meat-production bovines, pigs and veal calves) and pathogenic *E. coli* (in pigs

and bovines) in Belgium are presented for the year 2011. For both groups, the same interpretative criteria were applied. Poultry and veal calf isolates of commensal *E. coli* demonstrated higher antimicrobial resistance prevalence than isolates from pigs and bovines. Fifty percent of broiler *E. coli* isolates were resistant to at least five antimicrobials, whereas sixty-one percent of bovine *E. coli* isolates were susceptible to all antimicrobials tested. On the other hand, bovine pathogenic *E. coli* isolates showed an extended resistance profile with more than half of the isolates being resistant to ten or more antimicrobials. The national monitoring results on commensal bacteria of pigs and broilers are not significantly different from the results from previously presented field studies on commensal bacteria of pigs and broilers in Belgium, although different methodologies of sampling and susceptibility testing were used.

Factors potentially contributing to fluoroquinolone resistance selection in commensal *E. coli* strains in poultry were studied through a series of in *vivo* experiments (**Chapter 5)**. The effect of the initial prevalence of enrofloxacin resistance in the *E. coli* gut microbiota, the effect of the bacterial fitness of the enrofloxacin-resistant strain and the effect of treatment with enrofloxacin (effect of dose and effect of route of administration) were assessed. Right after hatching, the chicks were inoculated either with a bacteriologically fit or a bacteriologically non-fit fluoroquinolone-resistant strain either as a minority or a majority of the total *E. coli* population. Six days later, the majority of chicks were treated for three consecutive days either orally or parenterally and using three different doses (under-, correct- and over-dose) of enrofloxacin. The results showed that fluoroquinolone resistance selection was influenced by treatment ($p<0.001$), bacterial fitness of the inoculation strain ($p<0.001$), administration route ($p=0.052$) and the interactions between bacterial fitness and administration route ($p<0.001$). The use of oral treatment seems to select more for fluoroquinolone

resistance, especially in the model where a non-fit strain was used for inoculation. Surprisingly no significant effect was seen for dosing indicating that the same resistance selecting effect is obtained independently from whether a half, correct or double dose is given.

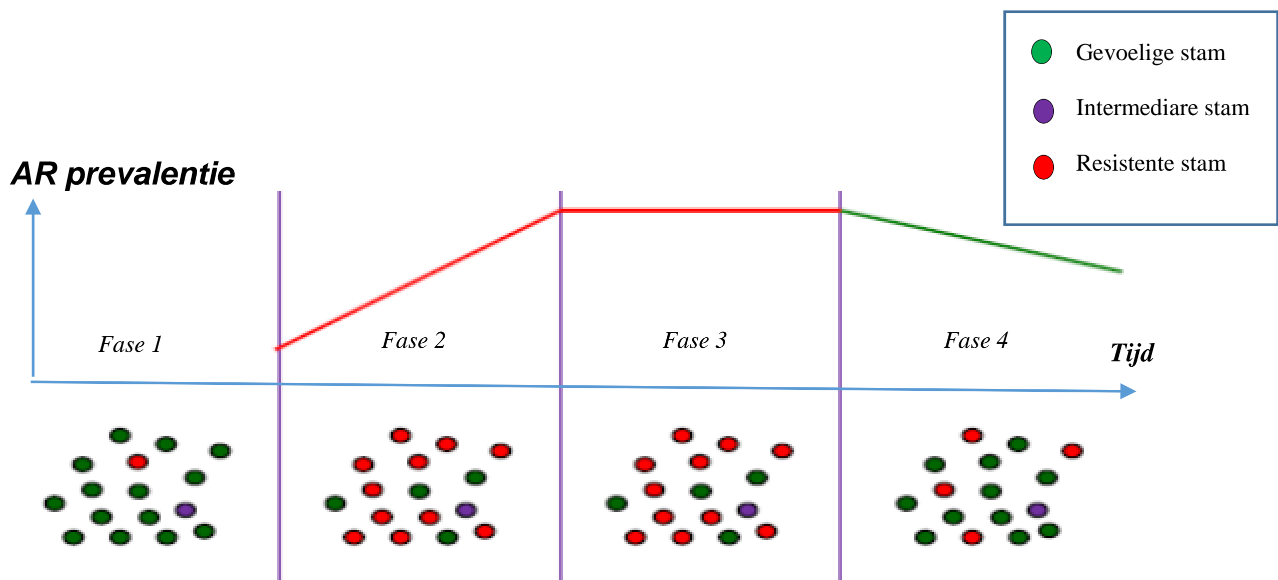
The effect of a competitive exclusion product (Aviguard®) on the spread of fluoroquinolone resistance in poultry was assessed *in vivo* in the absence or presence of fluoroquinolone treatment **(Chapter 6)**. A controlled seeder-sentinel animal model with one-day-old chicks was used. In experiment 1, as soon as the chicks were hatched, two groups were treated with a commercial competitive exclusion product (Aviguard®) and two were left untreated. Three days after hatching, 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, an identical experimental setup was used, but additionally all groups (except the control groups) were orally treated for three consecutive days (Day 8 -10) with enrofloxacin. The use of Aviguard® significantly ($p<0.01$) reduced the spread 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 spread of enrofloxacin resistance when no treatment was administered. Whereas this effect was no longer present when enrofloxacin was administered ($p = 0.70$).

CHAPTER 8.

SAMENVATTING

Nederlandse Samenvating

Resistentie tegen antimicrobiële agentia is tegenwoordig een van de grootste bedreigingen voor de wereldwijde gezondheid, voedselveiligheid, en ontwikkeling. Brede erkenning van dit wereldwijde probleem door de wetenschap en het publiek heeft geleid tot een toename in het aantal studies om meer inzicht te verkrijgen in de oorsprong en mechanismen van antimicrobiële resistentie. Daarnaast heeft het ook geleid tot de oprichting van verscheidene nationale surveillance programma's om het antimicrobiële gebruik te meten in de humane en veterinaire geneeskunde en het niveau van antimicrobiële resistentie in commensale bacteriën in zowel mensen als voedselproducerende dieren te monitoren. Echter, is er nog steeds sprake van een gebrek aan kennis over de epidemiologie van antimicrobiële resistentie en de factoren die bijdragen aan de spreiding en selectie van antimicrobiële resistentie op dier- en populatieniveau.



Figuur 1. Epidemiologie van antimicrobiële resistentie (AR). Vier fasen zijn beschreven: Fase 1. Ontstaan van AR, Fase 2. Selectie en spreiding van AR, Fase 3. Persistentie van AR, Fase 4. Reductie van AR. Tijdens Fase 1 is de meerderheid van de stammen gevoelig. Als er sprake is van selectiedruk (e.g. gebruik van antimicrobiële middelen) neemt het aantal gevoelige stammen af en komen de resistente stammen in de meerderheid (Fase 2) en zullen persisteren (Fase 3). Bij afwezigheid van selectiedruk kan een vermindering van de prevalentie van AR plaatsvinden (Fase 4).

De epidemiologie van antimicrobiële resistentie, alhoewel complex, kan in grote lijnen beschouwd worden als een opeenvolging van vier fasen (Fig. 1). De algemene introductie van **Hoofdstuk 1.1.** bespreekt de belangrijkste aspecten van de selectie, spreiding en persistentie van AR (fasen 2 en 3) door het gebruik antimicrobiële middelen in de veehouderij. Echter, naast het gebruik van antimicrobiële middelen kunnen andere factoren ook een rol spelen in de selectie en spreiding van antimicrobiële resistentie determinanten. Daarom wordt in **Hoofdstuk 1.2.** een gedetailleerd overzicht gegeven van alle belangrijke niet-antimicrobiële factoren die mogelijk de selectie en spreiding van antimicrobiële resistentie kunnen beïnvloeden.

Gebaseerd op data van publiekelijk verkrijgbare nationale of internationale monitoringsrapporten van zeven Europese landen, worden correlaties tussen het gebruik van antimicrobiële middelen in voedselproducerende dieren en de prevalentie van resistente commensale *Escherichia coli* isolaten afkomstig van varkens, pluimvee en rundvee, voor acht verschillende antimicrobiële agentia, geëvalueerd in **Hoofdstuk 3**. De relatie tussen de hoeveelheid antimicrobiële middelen toegediend aan voedselproducerende dieren per land (uitgedrukt in mg/populatie correctie eenheid) en de prevalentie van antimicrobiële resistentie (geïnterpreteerd door EUCAST epidemiologische cut-off waarden) in *E. coli* isolaten (4831 isolaten in totaal) voor antimicrobiële agentia representatief voor de verschillende gebruikte antimicrobiële klassen, werd onderzocht. Voor alle onderzochte antimicrobiële klassen werden opvallend hoge correlatiecoëfficiënten verkregen, wat aantoont dat op nationaal niveau de mate van het gebruik van specifieke antimicrobiële middelen sterk gecorreleerd is aan het niveau van resistentie tegen deze agentia in commensale *E.coli* isolaten bij varkens, pluimvee en rundvee. Echter, databeperkingen laten de noodzaak zien van een gedetailleerdere uitwerking van de verzameling en

harmonisatie van data met betrekking tot het gebruik van antimicrobiële middelen en antimicrobiële resistentie in Europa.

In **Hoofdstuk 4** worden gedetailleerde studies gepresenteerd over antimicrobiële resistentie van commensale *E. coli* (bij vleeskuikens, vlees producerende runderen, varkens en vleeskalveren) en pathogene *E. coli* (bij varkens en runderen) in België voor het jaar 2011. Voor beide groepen werden dezelfde interpretatieve criteria toegepast. Bij commensale *E. coli* isolaten afkomstig van pluimvee en vleeskalveren werd een hogere prevalentie van antimicrobiële resistentie vastgesteld dan bij isolaten van varkens en runderen. Vijftig procent van de *E. coli* isolaten van vleeskuikens was resistent tegen ten minste vijf antimicrobiële middelen, terwijl eenenzestig procent van de *E. coli* isolaten van runderen gevoelig was voor alle geteste antimicrobiële middelen. Daarentegen vertoonden pathogene *E. coli* isolaten van runderen een uitgebreid resistentieprofiel waarbij meer dan de helft van de isolaten resistent was tegen tien of meer antimicrobiële middelen. De resultaten afkomstig van de nationale monitoring van commensale bacteriën bij varkens en vleeskuikens zijn niet significant verschillend van de resultaten van eerder gepubliceerde studies over commensale bacteriën bij varkens en vleeskuikens in België, hoewel verschillende methodes van bemonstering en gevoeligheidstesten zijn gebruikt.

Factoren die mogelijk bijdragen aan de resistentieselectie voor fluoroquinolonen in commensale *E. coli* stammen bij pluimvee werden bestudeerd met behulp van een serie *in vivo* experimenten (**Hoofdstuk 5**). Het effect van de initiële prevalentie van enrofloxacine resistentie in *E.coli* in de darmmicrobiota, het effect van de bacteriële fitness van enrofloxacine resistente stammen en het effect van een behandeling met enrofloxacine (effect van de dosis en het effect van de toedieningswijze) werden beoordeeld. Onmiddellijk na het uitbroeden werden de kuikens geïnoculeerd hetzij met

een bacteriologisch “fit” of een bacteriologisch “non-fit” fluoroquinolone-resistente stam ofwel als een minder- of meerderheid van de totale *E. coli* populatie. Zes dagen later werd de meerderheid van de kuikens behandeld voor 3 opeenvolgende dagen, oraal of parenteraal, en met drie verschillende doseringen enrofloxacine (onder-, correct of overgedoseerd). De resultaten toonden aan dat de fluoroquinolone resistentieselectie erg beïnvloed werd door de behandeling ($p<0.001$), de bacteriële fitness van de geïnoculeerde stam ($p<0.001$), en matig beïnvloed door de toedieningswijze ($p=0.052$) en de interacties tussen de bacteriële fitness en toedieningswijze ($p<0.001$). Een orale behandeling leek meer te selecteren voor fluoroquinolone resistentie, met name in het model waarbij een “non-fit” stam werd gebruik voor inoculatie. Verrassend genoeg werd er geen significant effect gezien bij de dosering, wat aangeeft dat hetzelfde resistentie selecterende effect wordt verkregen onafhankelijk van de toediening van een halve, correcte of dubbele dosis.

Het effect van een competitief exclusie product (Aviguard®) op de spreiding van fluoroquinolone resistentie in pluimvee werd *in vivo* beoordeeld bij de af- of aanwezigheid van een behandeling met fluoroquinolonen (**Hoofdstuk 6**). Een gecontroleerd seeder-sentinel diermodel met eendagskuikens werd hiervoor gebruikt. In experiment 1, op het moment dat de kuikens waren uitgebroed, werden twee groepen behandeld met een commercieel competitief exclusie product (Aviguard®) en twee groepen werden niet behandeld. Drie dagen na het uitbroeden werden alle groepen geïnoculeerd met een enrofloxacine-gevoelige commensale *E. coli* stam. Vijf dagen na het uitbroeden werden twee dieren per groep geïnoculeerd met of een bacteriologisch “fit” of een bacteriologisch “non-fit” enrofloxacine-resistente commensale *E. coli* stam. In experiment 2 werd een identieke experimentele opzet gebruikt, maar daarnaast werden alle groepen (behalve de controle groepen) oraal

behandeld met enrofloxacine voor drie opeenvolgende dagen (Dag 8-10). Het gebruik van Aviguard® reduceerde significant ($p<0.01$) de spreiding van enrofloxacine-resistente *E. coli* wanneer de dieren niet met enrofloxacine waren behandeld. Daarnaast had ook de bacteriële fitness van de enrofloxacine-resistente *E.coli* stam gebruikt voor inoculatie een effect ($p<0.01$) op de spreiding van de enrofloxacine resistentie wanneer er geen behandeling was toegediend. Terwijl dit effect niet meer aanwezig was wanneer enrofloxacine wel was toegediend ($p = 0.70$).

DANKWOORD

Dankwoord

Σα βγεις στον πηγαιμό για την Ιθάκη,
να εύχεσαι νάναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
(Ιθάκη, Οκτώβρης 1911)
Κ.Π. Καβάφης

As you set out on the way to Ithaca
hope that the road is a long one,
filled with adventures, filled with understanding.
Ithaca, October 1911
C.P. Cavafy

These were the words that kept coming on my mind when I started my journey to Ghent. I had just left my beloved village –Aiani- and pretty much my life up to then and ventured forth to fulfil my everlasting dream; to do research. It goes without saying that my first thanks go to Jeroen, the person that accepted me to join the Veterinary Epidemiology Unit. Jeroen, you have been a constant source of motivation for me and your guidance has helped me not only on the epidemiological field but also on so many others. Your charisma to break down complex processes or concepts into comprehensible and meaningful information is unique. Your academic contribution is already significant and I am sure you will keep reaching new heights. Filip, I will never forget your help. You have been always there for me, saying always the appropriate words and making the correct remarks to keep me afloat. You are a bright and knowledgeable scientist and more importantly a great person to work with. The future belongs to you! Annemieke, thank you very much for providing your input and your constructive comments. I wish you success with your well-deserved position on UAntwerpen. I am sure you will have a big scientific career! My dear promotors, my gratitude to you will be endless and I would be glad to collaborate with you on any given day. I wish the best for you and your families!

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CURRICULUM VITAE

Curriculum Vitae

Ilias Chantziaras was born on October 21 1982 in Kozani, Greece. He is a veterinarian who graduated at the Faculty of Veterinary Medicine, University of Thessaly, Greece. In 2008, he successfully finished a post-graduate training in the Laboratory of Foods of Animal Origin and Public Hygiene in the same faculty. He has worked for the Greek Organization of Milk and Meat, the Greek Payment Authority of Common Agricultural Policy (C.A.P.) Aid Schemes, the Greek Army and as a private veterinarian. Since 2012, he is a member of the Epidemiology Unit, Department of Reproduction, Obstetrics and Herd Health, Ghent University. Since April 2013, and in collaboration with the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, he has been working on a PhD project focusing on the epidemiology of antimicrobial resistance in commensal *E. coli* in farm animals. This four year research project was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment. Furthermore, he is a resident for the European College of Veterinary Public Health.

During his PhD he has presented the results of his research in various national and international scientific conferences.

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1. 30 June 2013 – 3 July 2013

AULA Ghent, Belgium

5th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE) 2013

"Linking antimicrobial use and antimicrobial resistance in veterinary medicine at national level: is there a measurable correlation? Report on 7 European countries"

Ilias Chantziaras, Filip Boyen, Bénédicte Callens, Jeroen Dewulf

2. 19 September 2013 – 20 September 2013

Turin, Italy

ECVPH Annual General Meeting & Annual Scientific Conference 2013

"Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries"

Ilias Chantziaras, Filip Boyen, Bénédicte Callens, Jeroen Dewulf

3. 15 October 2015- 16 October 2015

Ghent, Belgium

Third Intestinal Health Scientific Interest Group (IHSIG) International

Symposium on Poultry Gut Health 2015

"Evaluation of the effect of route of administration and treatment dose on fluoroquinolone resistance in E. coli in broilers when using strains of different bacteriological fitness"

Ilias Chantziaras, Filip Boyen, Annemieke Smet, Jeroen Dewulf

Poster presentations in international and national conferences:

1. 30 June 2013 – 03 July 2013

AULA Ghent, Belgium

5th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE) 2013

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3. 8 November 2013

DGZ-Vlaanderen, Drongen , Belgium

20th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics

"Antimicrobial resistance prevalence among pathogenic- commensal *Escherichia coli* from food producing animals in Belgium"

Ilias Chantziaras, Patrick Butaye, Filip Boyen, Bénédicte Callens, Jeroen Dewulf

4. 26 March 2014- 28 March 2014

Dublin, Ireland

Annual conference of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM)

"Comparing antimicrobial resistance prevalence studies on pathogenic-commensal *E. coli* from pigs and bovines in Belgium"

Ilias Chantziaras, Patrick Butaye, Filip Boyen, Bénédicte Callens, Jeroen Dewulf

5. 29 June 2015- 01 July 2015

Tours, France

6th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE)

"Evaluation of the effect of administration and treatment dose on fluoroquinolone resistance in *E. coli* in broilers"

Ilias Chantziaras, Filip Boyen, Annemieke Smet, Jeroen Dewulf

6. 07 October 2015 – 09 October 2015

Belgrade, Serbia

European College of Veterinary Public Health (ECVPH) annual conference

"Evaluation of the effect of treatment, administration route and treatment dose on fluoroquinolone resistance in *E. coli* in broilers"

Ilias Chantziaras, Filip Boyen, Annemieke Smet, Jeroen Dewulf

7. 07 October 2015 – 09 October 2015

Belgrade, Serbia

European College of Veterinary Public Health (ECVPH) annual conference

“Evaluation of the effect of route of administration and treatment dose on fluoroquinolone resistance in *E. coli* in broilers when using strains of different bacteriological fitness”

Ilias Chantziaras, Filip Boyen, Annemieke Smet, Jeroen Dewulf

8. 30 October 2015

Melle, Belgium

Flemish Society for Veterinary Epidemiology & Economics (FSVEE) study day

Evidence-Based Veterinary Medicine (EBVM): Epidemiologie in Dienst van de Praktijk

“Evaluation of the effect of route of administration and treatment dose on fluoroquinolone resistance in *E. coli* in broilers when using strains of different bacteriological fitness”

Ilias Chantziaras, Filip Boyen, Annemieke Smet, Jeroen Dewulf