UNIVERSITEIT GENT

FACULTEIT DIERGENEESKUNDE
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EFFICACY OF FLORFENICOL AND GAMITHROMYCIN IN AN *ORNITHOBACTERIUM RHINOTRACHEALE* INFECTION MODEL IN TURKEYS

A PHARMACOKINETIC/PHARMACODYNAMIC INTEGRATION

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Thesis submitted in fulfillment of the requirements for the degree of
Doctor in Philosophy (PhD) in Veterinary Science
2016

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Efficacy of florfenicol and gamithromycin in an *Ornithobacterium rhinotracheale* infection model in turkeys: a pharmacokinetic/pharmacodynamic integration

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Cover design and printing by University Press, Zelzate

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The most beautiful experience we can have is the mysterious.
It is the fundamental emotion which stands at the cradle of true art and true science.
Albert Einstein

LIST OF ABBREVIATIONS

ACN	acetonitrile
ADME	absorption, distribution, metabolism and excretion
AMCRA	Antimicrobial Consumption and Resistance in Animals
ANOVA	analysis of variance
APV	avian metapneumovirus
AUC_{inf}	area under the curve from time 0 to infinity
AUC_{last}	area under the curve from time 0 to the last time point with a quantifiable concentration
BRD	bovine respiratory disease
BW	body weight
cfu	colony forming units
CAP	chloramphenicol
CAT	chloramphenicol acetyltransferase
CD_{50}	ciliostatic dose
Cl	total body clearance
CLSI	Clinical Laboratory Standard Institute
C_{max}	maximum plasma concentration
CO_{CL}	clinical cut-off value
$CO_{PK/PD}$	pharmacokinetic/pharmacodynamic cut-off value
CO_{WT}	epidemiological cut-off value
CYP450	cytochrome P450 enzyme complex
ED	efficacious dose
EC	efficacious plasma concentration
EUCAST	European Committee on Antimicrobial Susceptibility Testing
F_{abs}	absolute bioavailability
FF	florfenicol
F_{rel}	relative bioavailability
g	goodness-of-fit coefficient
g	gravity
GAM	gamithromycin
GFR	glomerular filtration rate
h-ESI	heated electrospray ionization

HPLC	high-performance liquid chromatography
IL-6	interleukin 6
IM	intramuscular
IS	internal standard
IV	intravenous
k_{abs}	absorption rate constant
k_{el}	elimination rate constant
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LOD	limit of detection
log P	octanol-water partition coefficient
LOQ	limit of quantification
m/z	mass-to-charge ratio
MeOH	methanol
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
MIC_{50}	MIC required to inhibit the growth of at least 50% of the evaluated organisms
MIC_{90}	MIC required to inhibit the growth of at least 90% of the evaluated organisms
MRL	maximum residue level
NaOH	sodium hydroxide
OIE	World Organisation for Animal Health
p.a.	post administration
p.b.i.	post bacterial infection
p.v.i.	post viral infection
PBS	phosphate-buffered saline
PD	pharmacodynamic
PELF	pulmonary epithelial lining fluid
PGE2	prostaglandin E2
PK	pharmacokinetic
pK_a	acid dissociation constant
PO	*per os*/oral
r	correlation coefficient
ROA	route of administration

RSD	relative standard deviation
SC	subcutaneous
SNGFR	single nephron glomerular filtration rate
SRD	swine respiratory disease
SRM	selected reaction monitoring
$t_{1/2abs}$	half-life of absorption
$t_{1/2el}$	half-life of elimination
TAP	tiamphenicol
t_{max}	time to maximum plasma concentration
TNF-α	tumor necrosis factor alpha
UHPLC	ultra-high performance liquid chromatography
V_d	volume of distribution
VetCAST	EUCAST Veterinary Subcommittee on Antimicrobial Susceptibility Testing
WHO	World Health Organisation

GENERAL INTRODUCTION

1. Respiratory infections in turkeys

1.1. Pathogens responsible for respiratory infections

Viral and bacterial infections of the respiratory tract often affect turkeys during the production round, resulting in economic losses due to an increased mortality and feed conversion rate, a reduction in growth rate and high medical costs (van Empel and Hafez, 1999). Besides viral and bacterial causes, respiratory disease in poultry is often a multifactorial problem and is frequently triggered by non-infectious factors such as poor management or housing conditions (temperature, ventilation, humidity, atmospheric ammonia and dust). These environmental factors may facilitate the pathogenic action of the pathogen, resulting in clinical symptoms (Glisson, 1998, 2013; Kleven, 1998).

Several avian viruses, including Newcastle disease virus, other avian paramyxoviruses (type 2, 4 and 6) and aviary influenza, infect the respiratory tract as well as other organs, whereas avian metapneumovirus (APV) and infectious laryngotracheitis virus only invade respiratory tissues (Villegas, 1998; Warke et al., 2008). APV, which belongs to the genus *Metapneumovirus*, is widely spread in the poultry industry, and is well known as turkey rhinotracheitis (Fig. 1) or "swollen head syndrome" in chickens, with swelling of the infraorbital sinuses being the most clearly visible symptom. Infection is reported in turkeys and chickens of all ages, but the most severe clinical signs are seen in young turkeys, broilers and heavy breeders during production. Vaccines are used to control the disease in chickens and turkeys (Villegas, 1998). The morbidity rate of APV is high (up to 100%) and the mortality rate depends on the age of the birds and the presence of concomitant infections with other

Figure 1. Clinical symptoms of avian metapneumovirus in turkey poults, with swollen infraorbital sinuses, nasal and ocular discharge, which can be mucopurulent in case of bacterial co-infection.

(secondary) agents, such as *Escherichia coli*, *Mycoplasma* spp., or *Ornithobacterium rhinotracheale* (Naylor et al., 1992; Hafez, 1998). These co-infections may bring increased condemnation.

Next to viruses, many bacterial pathogens are involved in respiratory diseases. *Pasteurella multocida* causes fowl cholera. Although the bacterium induces purulent exudative lesions in multiple organ systems, the respiratory pathology is the most important aspect of the disease (Glisson, 1998). Infectious coryza is caused by *Avibacterium paragallinarum* and infects the upper respiratory tract of chickens with symptoms of swollen infraorbital sinuses, nasal discharge and depression (Glisson, 1998). To prevent fowl cholera and infectious coryza, vaccines are available. Bordetellosis (*Bordetella avium*) is referred to as turkey coryza, as the clinical signs are similar to infectious coryza in chickens. However, the disease is more severe in turkeys compared to chickens. Young turkeys show sneezing, oculonasal discharge, mouth breathing, tracheal collapse and reduced growth. Both vaccination and antimicrobial treatment have limited success (Glisson, 1998). *E. coli* is an ubiquitous bacterium in the gastro-intestinal tract of poultry, although it can give rise to secondary respiratory infections subsequent to viral or bacterial infections or environmental insult. Colibacillosis lesions are present in respiratory tissue, but also in pericardial sac and peritoneal cavities. Mycoplasmas cause respiratory disease in avian species. They tend to be host-specific, like *M. meleagridis* only infects turkey and *M. gallisepticum* may infect many species of birds, but these are not known to infect mammalian or other species (Kleven, 1998). In chickens, Mycoplasmas cause a chronic respiratory disease and in turkeys an infectious sinusitis (Chin, 2013; Raviv and Ley, 2013). Many studies proved the interaction between Mycoplasmas and other respiratory viruses and bacteria (Naylor et al., 1992; Kleven, 1998; Marien, 2007). Also *Chlamydia psittaci* is associated with respiratory distress in turkeys, alone or in combination with other pathogens (Vanrompay et al., 1997; Van Loock et al., 2005). Beside *E. coli*, *O. rhinotracheale* is another highly prevalent bacterium which causes secondary respiratory problems in turkey poults, with a high economic impact due to high medical costs, increased mortality and carcass condemnation rates or decreased growth and hatchability or drops in egg production. Therefore, this pathogen will be described more in detail in the following paragraph.

1.2. *Ornithobacterium rhinotracheale* infection

1.2.1. Characteristics of Ornithobacterium rhinotracheale

O. rhinotracheale is a Gram-negative, nonmotile, pleomorphic, rod-shaped, nonsporulating bacterium (Chin et al., 2013). It was characterized in 1991 from isolates of turkey, chicken and rook out of Europe and South Africa (van Beek et al., 1994; Vandamme et al., 1994). Nowadays this bacterium can be found worldwide in different avian species, including chicken, duck, falcon, goose, guinea fowl, gull, ostrich, partridge, pheasant, pigeon, quail, rook and turkey. Between those different isolates of *O. rhinotracheale*, pathogenicity differences exist (Chin et al., 2013).

O. rhinotracheale develops pin-point colonies (1-2 mm after 48h of incubation), which are grey to grey-white, circular and convex with an entire edge. *In vitro*, the bacterium grows on a common, nonselective blood agar, in air enriched with 5 – 10% CO_2 (Chin et al., 2013). To date, 18 serotypes of *O. rhinotracheale* are determined (serotype A to R). Host specificity of the serotypes has not been described, but 61% of the isolates in turkeys belong to serotype A (Chin et al., 2013). Also small-colony variants of *O. rhinotracheale* exist. They are characterized by low growth rates, an atypical colony morphology (Zahra et al., 2013).

O. rhinotracheale can spread by horizontal transmission, direct or indirect through aerosol and drinking water, and by vertical transmission, *in ovo* (van Empel and Hafez, 1999, Chin et al., 2013). This rapid transmission in combination with the difficulty to eradicate has led to an endemic infection of *O. rhinotracheale* worldwide, especially in countries with intensive poultry production (Hafez, 2002; Gornatti Churria et al., 2012). In Belgium, 40 commercial layer hen flocks were all positive for *O. rhinotracheale* and with high titers of the individual birds (Vandekerckhove et al., 2004). Also in hobby poultry, the seroprevalence of *O. rhinotrachele* is very high. In a study of Haesendonck et al. (2014), all of 56 tested flocks were positive.

Notwithstanding many studies about *O. rhinotracheale* infections in poultry have already been performed, very little is known about interactions with avian hosts. Tabatabai et al. (2010) observed that the majority of the North American field isolates of *O. rhinotracheale* showed β-hemolytic reactions. This haemolytic activity may contribute to the virulence of the bacterium. Another virulence factor that is characterised in *O. rhinotracheale* is neuraminidase enzymatic activity. This NanO neuraminidase can liberate sialic acid from

poultry glycoproteins. But it is not known whether NanO of *O. rhinotracheale* has a role in host tissue colonization or inflammation (Kastelic et al., 2013). A lot of pathogens use iron acquisition mechanisms to conquer the host's capacity to limit available iron during the infection process. *O. rhinotracheale* also has this mechanism and this occurs via the iron-bound protein pathway rather than through the siderophore secretion pathway (Tabatabai et al., 2008).

1.2.2. Clinical symptoms

Clinical signs occur at 2 to 4 days post infection (Van Empel et al., 1996). At this early stage, the bacterium is attached to cilia of the epithelium on the respiratory side of the air sacs. Later, bacterial cells and cell fragments can be found in macrophages in the airsacs and lungs (van Empel and Hafez, 1999).

The severity, duration and mortality of an *O. rhinotracheale* infection are variable and depend on the strain, co-infection and environmental factors. In turkeys, an age difference has been reported, where older turkeys have a higher severity of clinical signs and mortality (Roepke et al., 1998). Although, mostly young poults of 2 to 8 weeks of age are infected (Chin et al., 2013). Mild symptoms are coughing, sneezing and nasal discharge and these can be followed by severe respiratory distress, dyspnea and sinusitis, resulting in reduction of feed and water consumption. Oedema, consolidation of the lungs with fibrinous exudates on the pleura (Fig. 2), airsacculitis, pericarditis, peritonitis and mild tracheitis are gross lesions that can be seen (Chin et al., 2013).

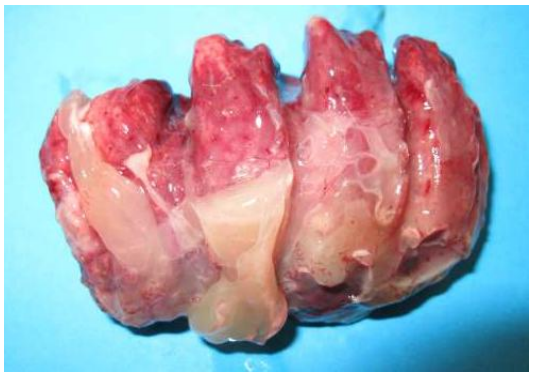

Figure 2. Severe lung inflammation, caused by *Ornithobacterium rhinotrachelae* (Hafez, 2014).

The bacterium can also distribute to other sites of the body resulting in local pathology, such as hepatitis, meningitis and joint-infections (Back et al., 1998; Zbikowski et al., 2013). *O. rhinotracheale* has a preference for the tibio-tarsal joint, resulting in lameness. In the field, this is a common problem, sometimes more visible than the respiratory signs. Lameness is more observed in male turkeys, due to their higher body weight.

1.2.3. Diagnosis and identification

Diagnosis of an *O. rhinotracheale* infection based on the clinical signs is difficult as there are a lot of agents, viruses and bacteria, resulting in a similar symptomatology. The differential diagnosis for *O. rhinotracheale* are other bacterial pathogens which cause respiratory lesions, such as *E. coli*, *P. multocida*, *A. paragallinarium* and *C. psittaci* (Chin et al., 2013). Isolation and identification of the causative pathogen are crucial for a correct diagnosis.

Trachea, lungs and air sacs are the best tissues in the respiratory tract to isolate *O. rhinotracheale*. But also swabs from the tibio-tarsal joint can be used for bacterial isolation. Due to overgrowth of other bacteria, *O. rhinotracheale* can be masked in samples of the infraorbital sinus and nasal cavity. To specify the growth on blood agar, gentamicin and/or polymyxin B can be added to the medium to inhibit overgrowth of fast-growing bacteria, such as *E. coli*, *Proteus* spp., *Pseudomonas* spp. (Garmyn et al., 2009). For identification of the bacterial strains, biochemical tests can be conducted. A combination of the agar gel precipitation test and the API-20NE identification strip (bioMérieux, France) is reliable to indentify *O. rhinotracheale*. These API strips give accurate identifications based on extensive databases and are standardized, easy-to-use test systems. The biochemical characteristics for *O. rhinotracheale* are: positive for oxidase, urease, β-galactosidase, arginine dehydrolase, alkaline phosphatase, esterase lipase, leucine arylamidase, cystine arylamidase, acid phosphatase, phosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase, and acid production from glucose, fructose, lactose and galactose. The bacterium is negative for nitrate reduction, catalase, growth on McConkey agar, motility, lysine decarboxylase, indole production, gelatinase, esterase, lipase, chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, and acid production from maltose, sucrose, fructose and ribose (van Empel and Hafez, 1999).

Furthermore, serology is very useful for flock monitoring. A drawback with this technique is the occurrence of positive samples due to maternal antibodies, which cannot be distinguished from antibodies derived from an infection. Commercial ELISAs have been developed using different serotypes and antigens of *O. rhinotracheale*, allowing the detection of several serotypes (Chin et al., 2013).

Antigen detection, by immunofluorescence antibody testing or immunohistochemical staining, or detection of a specific genome sequence by PCR can be conducted for the identification of *O. rhinotracheale*. These procedures are able to identify a higher percentage of *O. rhinotracheale* infected animals compared to serology and bacteriology (Chin et al., 2013).

Also the resistance of an *O. rhinotracheale* strain can be determined with the above-mentioned techniques. Diffusion-based (with disk diffiusion or gradient strips) or dilution-based gives a direct result to what extent the pathogen is resistant or susceptible for the tested antimicrobials. The occurrence of resistance genes, investigated by PCR, can give more information about the resistance mechanism.

1.2.4. Treatment and control

1.2.4.1. Management and housing conditions

To control *O. rhinotracheale* infections in poultry-rearing, a strict biosecurity level is required as *O. rhinotracheale* appears to spread by direct and indirect contact through aerosols and drinking water (Chin et al., 2013). Moreover, environmental factors, such as poor management, inadequate ventilation, poor hygiene, high flock density, high ammonia level, incorrect temperature and relative humidity, can exert the pathogenic action of *O. rhinotracheale* (van Empel and Hafez, 1999). Consequently, optimal environmental conditions are necessary.

1.2.4.2. Antimicrobial treatment

Despite antimicrobials are frequently applied during *O. rhinotracheale* outbreaks, consideration about the choice of the antimicrobial agent is important as a high resistance level against a wide range of antimicrobial classes employed to treat *O. rhinotracheale* has been reported (van Veen et al., 2001; Soriano et al., 2003; Zaini et al., 2008). A standard procedure for susceptibility testing for *O. rhinotracheale* has not been established by the Clinical Laboratory Standard Institute (CLSI). However, several *in vitro* studies have been performed to investigate the susceptibility or resistance of *O. rhinotracheale* against many

antimicrobials. The used testing methods and criteria for susceptibility and resistance may differ among reports.

All strains were resistant to the aminoside antibiotics neomycin and gentamicin, but if this resistance is intrinsic is not known (Ak and Turan, 2001; van Veen et al., 2001; Banani et al., 2004). For the tetracyclines oxytetracycline and doxycycline and several fluoroquinolones (enrofloxacin, danofloxacin, flumequin), the susceptibility was strain dependent (Devriese et al., 1995; Ak and Turan, 2001; Devriese et al., 2001; Banini et al., 2004). The susceptibility of *O. rhinotracheale* against amoxicillin decreased (van Veen et al., 2001), whereas there was resistance against other β-lactam antibiotics (namely ampicillin, ceftiofur) (Devriese et al., 2001). Data about the first generation macrolides, like erythromycin and tylosin, show resistance against *O. rhinotracheale* strains (Devriese et al., 1995; Ak and Turan, 2001; van Veen et al., 2001; Banini et al., 2004). Accordingly, the sensitivity of *O. rhinotracheale* to above mentioned antimicrobial drugs is very inconsistent and strain-dependent. However, more recently introduced antibiotics, such as the newer macrolides, might have higher sensitivity to *O. rhinotracheale*.

According to the antibiotic guide to promote well-considered usage of antibiotics in poultry, published by the Belgian Institute of Antimicrobial Consumption and Resistance in Animals (AMCRA), the combination of trimethoprim and sulphonamides is the first choice to treat an infection with *O. rhinotracheale* (AMCRA, 2013). However, several studies confirmed the high resistance of *O. rhinotracheale*, isolated from farm, backyard and wild birds, against potentiated sulphonamides (Malik et al., 2003; Soriano et al., 2003; Szabo et al., 2015). Also tetracyclins (e.g. doxycycline and chlortetracycline) and amoxicillin can be administered, but as already mentioned, high levels of resistance against these antimicrobials have been reported too. Within the group of tetracyclines, doxycycline is preferable because of the higher oral bioavailability compared to chlortetracycline (AMCRA, 2013).

Only a few studies evaluated the efficacy of antimicrobial drugs administered in an *in vivo* infection model against *O. rhinotracheale* in turkeys. Marien et al. (2006) concluded that continuous drinking water medication with enrofloxacin (10 mg/kg body weight, BW) was the most successful drug, followed by florfenicol (FF, 20 mg/kg BW), whereas amoxicillin (20 mg/kg BW) was ineffective. Other studies investigated the efficacy of different enrofloxacin treatment regimens, in which the treatment of 10 mg/kg during 5 days gave the best results

(Garmyn et al., 2009a,b). To the author's knowledge, no other *in vivo* efficacy studies with *O. rhinotracheale* in turkeys have been performed.

1.2.4.3. Vaccination

To reduce treatment of infections by antimicrobials, vaccines can be administered to provide the host a complete or partial protection. For infections with *O. rhintracheale*, this is probably the best option as infections are endemic and difficult to eradicate, and the resistance level against several antimicrobials is high.

Despite these arguments, there is currently only one commercial vaccine against *O. rhinotracheale* registered in Europe, namely Nobilis OR Inac. This vaccine contains an inactivated whole cell suspension of *O. rhinotracheale* serotype A, strain B3263/91 (EMA, 2008a). It is used to vaccinate female chickens that are being used for breeding broilers, resulting in immunity of the progeny, which is a passive immunization. The vaccination scheme is as follows: a first injection (0.25 mL) at the age of 6 to 12 weeks and a second injection (2.5 mL) at least six weeks later (at the age of 14-18 weeks). The breeders can transfer the immunity to their offspring untill 43 weeks after the last vaccination. However, the administration of this vaccine to turkeys is off-label.

A lot of studies has been performed with a wide range of vaccines, such as bacterins, live vaccines and subunit recombinant vaccines, with variable results (Churria et al., 2013).

After immunization with the commercial monovalent vaccine Nobilis OR Inac, the performances of the chickens was better, with lower mortality and a higher production index (Cauwaerts et al., 2002; Bisschop et al., 2004; De Herdt et al., 2012). The inactivated vaccines are mostly serotype specific. Consequently, they are not effective as most outbreaks involve a mix of serotypes (van Empel and Hafez, 1999; Salmon and Watts, 2000; Schuijffel et al., 2006; Murthy et al., 2007). Therefore, an immunization strategy that protects birds from infections with heterologous serotypes is needed. Erganiş et al. (2010) prepared a bivalent bacterin with both serotype A and B. They observed an increased weight gain, better feed conversion ratio and lower morbidity and mortality of the vaccinated turkeys compared to non-vaccinated turkeys.

Vaccination with live vaccines of *O. rhinotracheale* is controversial because of the severity of an *O. rhinotracheale* infection, but is generally of higher quality than vaccination with killed whole-cell vaccines. Lopes et al. (2002) developed a live vaccine based on a temperature sensitive mutant strain of *O. rhinotracheale*, which is inactive at a temperature of 41 °C. This strain has the ability to colonize and persist in the upper respiratory tract, where it simulates the local immunity. As a result, the severity of an infection with *O. rhinotracheale* minimizes. Also the combination of a live vaccine and a bacterin would be efficacious to reduce symptoms induced by the live vaccine (van Empel and van den Bosch, 1998).

A subunit recombinant vaccine with recombinant proteins of a serotype G strain, gives cross-protection against A, B and G (Schuijffel et al., 2005; 2006).

2. Drug administration in poultry

2.1. Antimicrobial use in poultry

Antimicrobial agents are of great importance in the poultry industry and they are used prophylactic, metaphylactic and therapeutic. A controversial use of antimicrobials in poultry is the subtherapeutic use of antimicrobials as growth promoters. This category is banned in Europe since 2006 (EU, 2006), but is still permitted in the United States and Canada (such as flavophospholipol, virginiamycin). Also prophylactic use of antimicrobials is under discussion nowadays (BEUC, 2015).

The preventive or prophylactic administration of antimicrobials takes place prior to the appearance of clinical signs of disease. The route of administration of prophylactic drugs depends on the age of the bird. The drug can be given *in ovo* or subcutaneously in 1-day-old chicks. For older animals, it can also be administered through the drinking water or feed. In contrast, the therapeutic use is intended to cure the birds from clinical disease. These drugs are mainly administered *via* the drinking water, as sick birds may refuse to eat but still drink. However, the boundary between prophylaxis and treatment is very narrow, as poultry are mainly treated on flock level. Since a house can contain more than 40,000 birds, a disease can spread very quickly. As a result, the preferred method to treat is group medication, in which all sick individuals and those in contact and at high risk of exposure (the entire flock) are treated (metaphylactic treatment) (Vermeulen et al., 2002; Hofacre et al., 2013). In case of infection, the veterinarian has to decide whether the birds can be treated with an antimicrobial, and, if so, which antimicrobial and by what route of administration (Hofacre, 2002). The veterinarian has to consider several factors including effectiveness against the pathogen, pharmacokinetic/pharmacodynamic (PK/PD) properties of the drug, withdrawal times, economics/cost-benefit, animal welfare and impact on the ability to market the final product (Hofacre et al., 2013). For the PK/PD properties, it should be mentioned that the susceptibility testing is usually performed on only one isolate of the pathogen, whereas poultry are often affected by several isolates of the same bacterial species, with a wide range of minimum inhibitory concentration (MIC) values. Moreover, these values are not uniform worldwide and have geographic variations (Salmon and Watts, 2000). Therefore, an

empirical treatment can be started, prior to the results of bacterial culture and susceptibility testing.

The reduction of the emergence of antimicrobial resistance implies a reduction in the inappropriate and excessive use and also the selection of the optimal drug, dose and duration of treatment (Landoni and Albarellos, 2015). Therefore, PK/PD studies are essential to realize this goal.

The AMCRA 2020 vision statement defines the guidelines of the policy relating to the use of antibiotics and to antibiotic resistance among animals in Belgium (AMCRA, 2015). The three main objectives about the consumption of antimicrobials are 1) a 50% reduction in antimicrobial use by 2020, 2) a 75% lower use of the most critical antimicrobials (cephalosporins and fluoroquinolones) by 2020 and 3) a 50% reduction in use of feed medicated with antibiotics by 2017. Also the poultry industry has to contribute to achieve these objectives.

As mentioned above, the preferred route of administration is oral treatment *via* feed or drinking water. Consequently, as little as 1% of the total pharmacotherapy of birds involves parental administration, which can be performed subcutaneaously (SC) at the neck or axilla and intramuscularly (IM) in the pectoral or leg muscle (Vermeulen et al., 2002). However, this method is very time consuming, stressful for the birds (individual handling) and can give rise to residues in edible tissues in case of off-label use. Some formulations result in a slow release with undesirable absorption patterns and possibly prolonged withdrawal times. Furthermore, IM administration can give rise to muscular damage, with economic losses. A comparison between oral (flock) and parenteral (individual) administered drugs is given in Table 1.

Table 1. Comparison between individual or flock treatment (adapted from Vermeulen et al., 2002).

Parenteral - individual	**Oral - flock treatment**
Advantages: - correct dosage - each bird - only sick animals	Advantages: - easy to perform - no tissue damage - better animal welfare
Disadvantages: - time and labour consuming - tissue necrosis and residues - less animal welfare (stressful)	Disadvantages: - global dosage - depends on feed and water intake - sick animals not always treated

2.2. Medication through drinking water and feed

As stated before, medication through drinking water and feed is most commonly applied in intensively reared poultry. Since an individual bird has a low economic value, parenteral administration is practically impossible. Sick birds show a significant reduction in their water and feed consumption, but the decline in drinking water uptake is usually less compared to feed.

To calculate the most accurate dose in medicated water or feed, the total body weight of birds in a flock should be taken into account. That dose should be included in the volume of water or feed the birds are expected to consume each dosing interval (Hofacre et al., 2013).

Drinking water medication offers several advantages but also some disadvantages. The low cost organization, the low work load and the ease of administration are very important factors for the farmer. An instant therapeutic effect and the possibility of a quick change of drug and/or dose have an influence on the birds' health (Vermeulen et al., 2002). On the other hand, a great disadvantage is the variability of drug intake, as a result of the individual animal (grade of sickness, pecking order), the individual farmer (unprofessional use or preparation of the solution), the drug properties (stability, solubility) and/or the drinking water quality (pH, hardness, nitrite and iron concentration, bacterial contamination, see Table 2). Medicated solutions should generally be replaced every 24 hours, although for some drugs, like β-lactam antimicrobials, this interval is shorter, depending on their stability.

A drug formulation needs to be physically and chemically stable over a sufficient period of time to allow a homogenous dose administration and consequently an efficient therapy. Therefore, an adapted formulation of the active substance is essential for medication through drinking water to help to improve solubility and stability of the drug. Beside a continuous administration, also a pulsed administration of medicated drinking water can be used, this for a limited period between 4 and 12 h, followed by administration of unmedicated water (Charleston et al., 1998). Drinking water medication is usually administered using a bulk tank, in which the entire volume of the water tank is filled with medicated water at the appropriate concentration. Another method is the water proportioner, which is a device that meters the drug from a highly concentrated stock solution to the drinking water, achieving the correct concentration (Hofacre et al., 2013). This concentrated solution may mount up to 100 fold the therapeutic concentration. This poses real challenges from a pharmaceutical technical point of view, in order to keep optimal solubility and dissolution of the concentrated drug.

Table 2. Standards for water quality for poultry in Belgium (adapted from *Dierengezondheidszorg Vlaanderen*, 2014) and results of water quality in poultry stable of Faculty of Veterinary Medicine.

Chemical	**Allowance in Belgium**	**Belplume**	**Faculty of Veterinary Medicine**
Physical aspect	Clear, colour- and odorless	Clear, colour- and odorless	Clear, colour- and odorless
pH	3.5 – 9	4 – 9	ND
Total hardness	-	≤ 20°D	32.2°D
Nitrite	≤ 1.0 mg/L	≤ 1.0 mg/L	< 0.1 mg/L
Total iron	≤ 2.5 mg/L	≤ 2.5 mg/L	< 0.025 mg/L
Bacteriological	**Allowance in Belgium**	**Belplume**	**Faculty of Veterinary Medicine**
Total plate count 22°C	≤ 1.10^6 cfu/mL	≤ 1.10^6 cfu/mL	480 cfu/mL
Total plate count 37°C	-	≤ 1.10^6 cfu/mL	ND
E.coli	≤ 1000 cfu/mL	≤ 100 cfu/mL	< 1 cfu/100 mL
Intestinal enterococci	≤ 1000 cfu/mL	< 1 cfu/100 mL	< 1 cfu/ 100 mL

Allowance in Belgium: according to KB 17 June 2013; Belplume is a quality system within the Belgian broiler chain

- : not specified; ND: not determined

Feed medication can be applied as a therapeutic as well as meta- or prophylactic treatment. In case of long term therapy of more than 5-7 days, feed medication is recommended since the flock begins to recover, resulting in more appetite. A major advantage of feed medication is the cost, since feed-grade antimicrobials are mostly less expensive than the water soluble alternatives (Hofacre et al., 2013). Medicated feed is manufactured by mixing feed with a premix formulation of a drug, resulting in ideally a stable and homogeneously distributed active substance. A high risk in the production of medicated feed is cross-contamination, with contamination of drugs to the next batch of feed. To reduce this cross-contamination with antimicrobials, mixing the premix at the end of the production process or using a fine dosage system during unloading at the farm, is obligatory in Belgium since 2014 (FAVV, 2013). Turkeys are often fed by pellets, and it is complicated to obtain a homogenous and thermostable product between the pellets and a premix. Other disadvantages of medicated feed are a limited flexibility in dosing, possibility of segregation and separation during transport, cross-contamination at the farm level, not advisable as start-up therapy, not very suitable for concentration-dependent antimicrobial products (such as aminoglycosides and fluoroquinolones). These properties can give rise to selection of antimicrobial resistance. Moreover, the oral bioavailability can be reduced as a result of interactions between the veterinary drug and some components of the feed, such as mycotoxin binders (Devreese et al., 2012; De Mil et al., 2015). As already reported, the aim is to reduce the use of medicated feed with 50% by 2017 (AMCRA, 2015).

2.3. Influence of environmental conditions

Since poultry are intensively reared, good husbandry practices are necessary for the poultry industry. To prevent sickness, the house environment is of great importance for animal health. Moreover, several environmental factors, such as water quality, photoperiod, ambient temperature and type of diet, can influence the treatment regime as they affect the water and feed consumption.

In general, the drinking water should be clean, cool and neutral in taste. The pH is an important parameter. The optimum pH of drinking water should range between 5 and 7. With a pH value below 5, the water consumption will decrease and it may lead to parasitic infestations (Vermeulen et al., 2002). However, the stability and solubility of certain antimicrobials like tetracyclines are optimal in water with a low pH (3-5). Santos et al. (1997) observed that the palatability of medicated water containing doxycycline and citric acid was good, as the water consumption of turkeys did not change significantly compared to non-medicated tap water. On the other hand, higher pH values may indicate possible contamination with salts (e.g. sodium bicarbonate), resulting in a lower utilization of dietary minerals, such as calcium, magnesium, phosphorus and potassium (Vermeulen et al., 2002). The combination of different drugs or the co-administration of a disinfecting product can result in incompatibilities and precipitation (Esmail, 1996). Also the water temperature is of importance as it determines the drug solubility and stability, especially for poor soluble drugs (Vermeulen et al., 2002).

Furthermore, the ambient temperature is related to the water intake. For broilers, the water consumption increases with 7% for every 1 °C above 21 °C. However, under extremely hot conditions, the water intake decreases as a result of the reduced activity of the birds (Vermeulen et al., 2002).

In addition, the eating and drinking patterns may alter depending on the light scheme (Classen et al., 1994), which can have a huge influence on the uptake of medicated drinking water. For time-dependent antibiotics, such as FF and gamithromycin (GAM) (Hesje et al., 2007), it is important to have a frequent drug-intake to obtain a time above the MIC (T>MIC) of minimum 40%. Birds exposed to a very short photoperiod (< 6 h light) as well as a continuous photoperiod seemed to have a reduced feed intake. The former can be due to a too short photoperiod for the higher diurnal rate of feeding to compensate for the reduced nocturnal feeding rate. The latter could be due to the absence of the need to store feed for a dark period (Lewis et al., 2009). Nowadays, a minimum of 6 h of light is required in poultry-rearing (Anonymous, 2007). Studies in chickens and turkeys have already related extreme photoperiods with a change in feeding cycles (Newberry, 1992; Brown et al., 2008; Lewis et al., 2009). Since feeding uptake in birds is strongly connected with water uptake, changes in photoperiod can also easily affect the drinking water uptake.

Finally, also the composition of the diet affects the water intake. Some protein sources, like soybeans, and a high content of fibers are positively correlated with the amount of water consumption, whereas a high energy diet requires less water uptake (Esmail, 1996).

In general, the environment might influence the feed and water uptake, and consequently also the drug intake, resulting in unexpected plasma concentrations and altered therapeutic outcome.

3. Principles of pharmacokinetics and pharmacodynamics of antimicrobials

To understand and control the efficacy of drugs in animals, one must know how much drug will reach the biophase and when this will occur. Especially for microbial infections, it is of great importance to rapidly reach effective concentrations of the antimicrobial drug at the site of infection, for an adequate duration (Baggott and Giguère, 2013).

Pharmacokinetics is a quantitative analysis of how man and animal handle xenobiotics, in other words what the body does to the drug (Mahmood, 2005). It includes absorption, distribution, metabolism and excretion of a drug in the body (ADME). The PD properties refer to the action of the drug in the body, including the therapeutic effects (Andes et al., 2004). Hence, the PK/PD approach gives an insight into the therapeutic action of antimicrobial drugs.

3.1. PK/PD indices

PK/PD-modeling combines two research fields in pharmacology and describes the effect-time course resulting from the administration of a certain dose of drug (Fig. 3). Furthermore, it can be used as a basis to predict the dose, dosing frequency and duration of drug administration (Martinez et al., 2013).

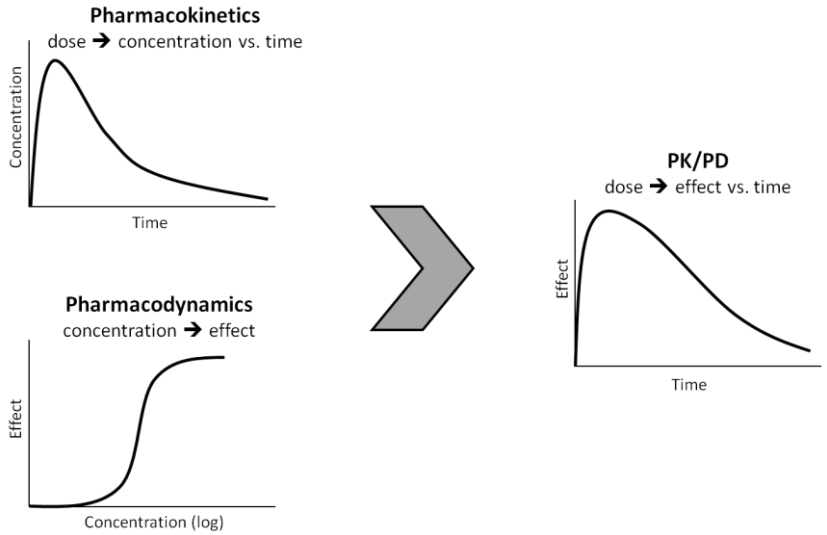

Figure 3. The relationship between pharmacokinetics (PK) and pharmacodynamics (PD), resulting in PK/PD-modelling (adapted from Meibohm and Derendorf, 1997).

Figure 4 shows the most widely used PK/PD indices for antimicrobial agents, which rely on plasma concentrations as PK parameter and the MIC as PD parameter (Giguère and Tessman, 2011). The efficacy of concentration-dependent antimicrobials, such as aminoglycosides, fluoroquinolones and metronidazole, is best predicted by correlating the maximum plasma concentration to the MIC (C_{max}/MIC ratio). β-lactams, macrolides and FF are examples of time-dependent antimicrobial agents. The efficacy of these drugs is associated with the time, expressed as a percentage of the dosage interval or maximum 24 h, that the concentration remains above the MIC (T>MIC). For antibiotics which are both concentration- and time-dependent, like azalides and tetracyclines, the ratio of the area under the curve (AUC) of a 24 h interval to MIC (AUC_{24h}/MIC) best predicts their efficacy (Giguère and Tessman, 2011; Mouton et al., 2012). Also for time-dependent drugs with a long post antibiotic effect, such as β-lactams and macrolides, AUC/MIC is the most appropriate PK/PD index (Munckhof et al., 1997; Martinez et al., 2013).

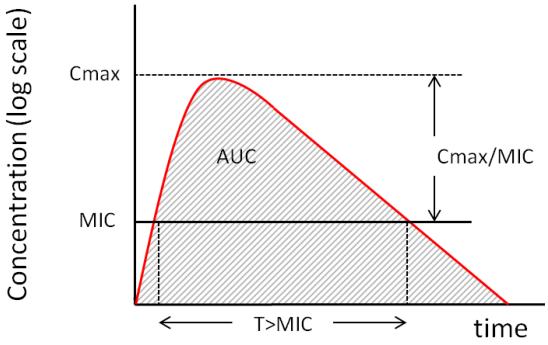

Figure 4. Pharmacokinetic (PK)-pharmacodynamic (PD) indices for antimicrobial drugs, with C_{max}, maximum plasma concentration; MIC, minimum inhibitory concentration of an antimicrobial; AUC, area under the plasma concentration-time curve; T>MIC, time the plasma concentration is above the MIC. For concentration-dependent antimicrobials, C_{max}/MIC is used to predict the efficacy, while T>MIC is associated with time-dependent antimicrobials. For antimicrobials which are both concentration-dependent and time-dependent, AUC/MIC is the most appropriate predictor for the efficacy.

3.2. Clinical breakpoints

It is generally assumed that effective antimicrobial therapy requires sustained blood or tissue concentrations above the MIC (Prescott and Baggott, 1994). Clinical breakpoints, established by the CLSI and the European Committee on Antimicrobial Susceptibility Testing

(EUCAST), are being compared with the acquired MIC values. These are indicators for the development of resistance and bacterial isolates can be categorized as susceptible, intermediate or resistant. CLSI and EUCAST define a microorganism as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success. A susceptible isolate of an infecting microorganism has a high chance to be successfully treated with the recommended dosage regimen of an antimicrobial agent. The term intermediate implies that an infection by the isolate can be treated at body sites where the drug is physiologically concentrated or when a high dosage is used. When an infecting isolate cannot be inhibited by typically achieved concentrations of an antimicrobial with normal dosage regimens and/or when clinical efficacy has not been reliable in field studies, the isolate can be considered as resistant (EUCAST, 2014). This classification can be used by clinicians to establish a treatment with an antimicrobial against the corresponding pathogen. The CLSI has already published some veterinary breakpoints, but to date, no clinical breakpoints for *O. rhinotracheale* in poultry have been determined yet.

An important consideration about the clinical outcome is the correlation between MIC, exposure and efficacy. The efficacy of an antimicrobial depends on the MIC against the pathogen and the exposure of that pathogen in the patient. In addition, the dose and the PK properties of a drug determine the exposure of the pathogen to the antimicrobial (Mouton et al., 2012).

3.3. Cut-off values

To determine clinical breakpoints, it is important to establish cut-off values. Turnidge et al. (2007) proposed that the term “cut-off” is more widely and describe the three types of “breakpoints”. On the other hand, the term “breakpoint” should be reserved for the final selected value to be applied in the clinical laboratory. In contrast with clinical breakpoints, cut-off values are no indicators for the development of resistance. The establishment of the clinical breakpoint can be based on three cut-off values, namely the epidemiological, the PK/PD and the clinical cut-off values.

First, the **epidemiological cut-off values** (CO_{WT}) are determined on the distribution of the MIC for an antimicrobial and a bacterium. When a population is clearly separated of the wild-type population, it can be categorized as non-wild type. The latter population consists of isolates with an acquired resistance mechanism to the drug (EUCAST, 2014). The epidemiological cut-off is set at the upper MIC value of the wild type distribution (Fig. 5). However, this parameter does not take into account the results of clinical efficacy studies, dosage and route of administration of the antimicrobial agent, or PK/PD indices in the animal species concerned (Silley, 2012).

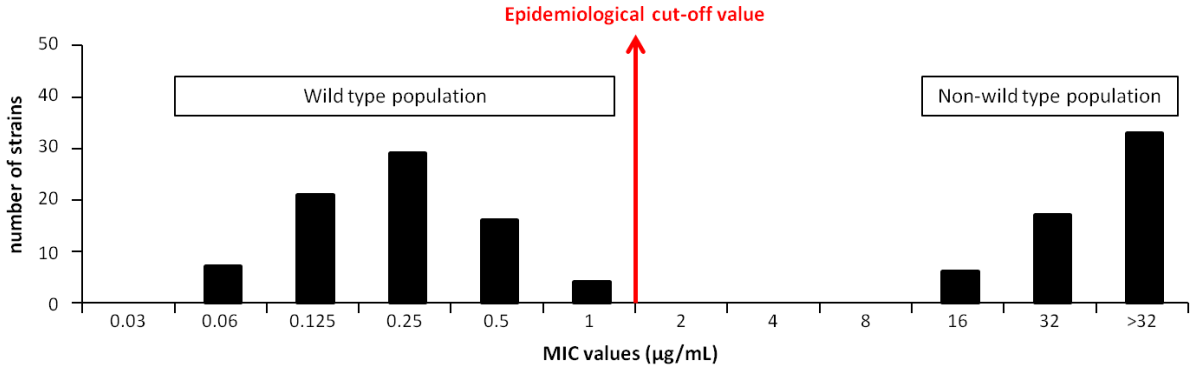

Figure 5. Epidemiological cut-off value. Results of a MIC determination of an antimicrobial agent "X" for several isolates of one bacterial species, with indication of the epidemiological cut-off value (adapted from Boyen et al., 2012).

Second, **PK/PD cut-off values** ($CO_{PK/PD}$) are based on the PK/PD indices T>MIC, AUC/MIC or C_{max}/MIC. Selecting the best suitable PK/PD index is essential to obtain PK/PD cut-offs. Table 3 gives an overview of several antimicrobial agents and their appropriate PK/PD indices.

There are absolute PK/PD cut-off values established for Gram-negative bacteria. These are a T>MIC of 40-50%, expressed for the dosage interval or a period of 24 h, an AUC/MIC of 125, based on a 24 h period in plasma in steady state condition, and a C_{max}/MIC between 8 and 10. For Gram-positive bacteria these cut-off values are determined at 40-50% and 30-50 for T>MIC and AUC/MIC, respectively (Hesje et al., 2007; Lees et al., 2008). However, for slowly eliminating antimicrobials or when no steady state situation is achieved, these cut-offs have no value. Martinez et al. (2013) suggested that the interval for T>MIC of these antibiotics may extend 24 h and the AUC_{inf} may be taken into account for plasma PK/PD correlations.

Table 3. Classification of antimicrobials according to type of killing action and their PK/PD indices (adapted from Lees et al., 2008).

Action types	Chemical groups	Drug examples	Integrated PK/PD variables correlating with bacteriological effect
Concentration-dependent killing, usually exerting significant post-antibiotic effect	Fluoroquinolones	Enrofloxacin, Danofloxacin, Marbofloxacin, Difloxacin, Ibafloxacin	AUC/MIC;C_{max}/MIC
	Aminoglycosides	Streptomycin, Neomycin, Gentamicin, Amikacin, Tobramycin	C_{max}/MIC
	Nitroimidazoles	Metronidazole	AUC/MIC;C_{max}/MIC
	Polymixins	Colistin	AUC/MIC
Time-dependent killing with either no or limited post-antibiotic effect	Penicillins	Benzylpenicillin, Cloxacillin, Ampicillin, Amoxicillin, Carbenicillin	T>MIC
	Cephalosporins	Ceftiofur, Cefalexin, Cefapirin	T>MIC
	Macrolides and triamilides	Aivlosin, Tylosin, Erythromycin, Tilmicosin, Tulathromycin	T>MIC[a]
	Lincosamides	Clindamycin	T>MIC
	Phenicols	Chloramphenicol, Florfenicol	T>MIC
	Sulfonamides	Sulfadoxine, Sulfadiazine	T>MIC
	Diaminopyrimidines	Trimethoprim	T>MIC
Co-dependent killing, that is killing action dependent on both duration of exposure and maintained drug concentration	Tetracyclines	Oxytetracycline, Chlortetracycline, Doxycycline	AUC/MIC
	Ketolides	Azithromycin, Clarithromycin	AUC/MIC
	Glycopeptides	Vancomycin	AUC/MIC

[a] For some macrolide and triamilide drugs, AUC/MIC best correlates with efficacy; for others no correlations have been established.

The afore-mentioned general PK/PD cut-offs are not specific for a particular pathogen-drug interaction. Therefore, VetCAST has proposed a step-wise calculation of this parameter. The first step to determine $CO_{PK/PD}$ is selecting the best suitable PK/PD index (Table 3). The second step is the determination of the critical value of the selected PK/PD index. This can be done by for example killing curve assay. The last step is the computation of the percentage of animals which are able to achieve the critical value of the selected PK/PD index, and this for a given animal species and for all possible MIC values. This can be executed by Monte Carlo simulations. The result of such simulations is the determination of the population distribution of doses that are able to achieve the critical value of the PK/PD index in the population taking into account the actual MIC distribution (Mouton et al., 2012; Toutain, 2015).

Both host and pathogen factors can affect the PK/PD results. In case of a normal immune system, an antimicrobial has to assist the animal to cure from the pathogen. Therefore, lower *in vivo* drug concentrations compared to MIC could still achieve recovery. Moreover, the site of infection can influence the drug efficacy. The current PK/PD indices are limited to plasma concentrations. However, drug concentration at the site of action can be quite different compared to plasma concentration, as demonstrated for the newer macrolides and quinolones. Therefore, PK results at the site of action, such as tissue homogenates, are more clinically relevant (Andes et al., 2004; Barbour et al., 2010; Giguère and Tessman, 2011). In contrast, Andes et al. (2004) stated that tissue homogenates may underestimate or overestimate the concentration in the interstitial space, because they contain the interstitial, intracellular and vascular compartments. Also the PD parameter can be influenced by the medium in which it is determined. The value of the MIC can be lower (for macrolides) or higher (tetracyclines) when measured in medium with serum, compared to artificial medium (Bruyck et al., 2012; Mitchell et al., 2013).
Furthermore, adapted cut-off values could be considered for some drugs as lung tissue and pulmonary epithelial lining fluid (PELF) concentrations of macrolides tend to be higher than plasma concentrations. Indeed, low plasma AUC/MIC ratios are still correlated with a good efficacy for telithromycin and azithromycin of only 3.38 and 5, respectively (Craig, 2001; Lodise et al., 2005; Martinez et al., 2013).

At last, the **clinical cut-off values** (CO_{CL}) reflect the upper limit of the MIC associated with a high likelihood of clinical success or the probability to cure. It is based upon the collection of isolates obtained during clinical effectiveness studies and there is no set method for establishing the CO_{CL} (Toutain, 2015).

To establish clinical breakpoints (susceptible, intermediate or resistant) out of these three cut-off values, a decision tree can be used (Fig. 6).

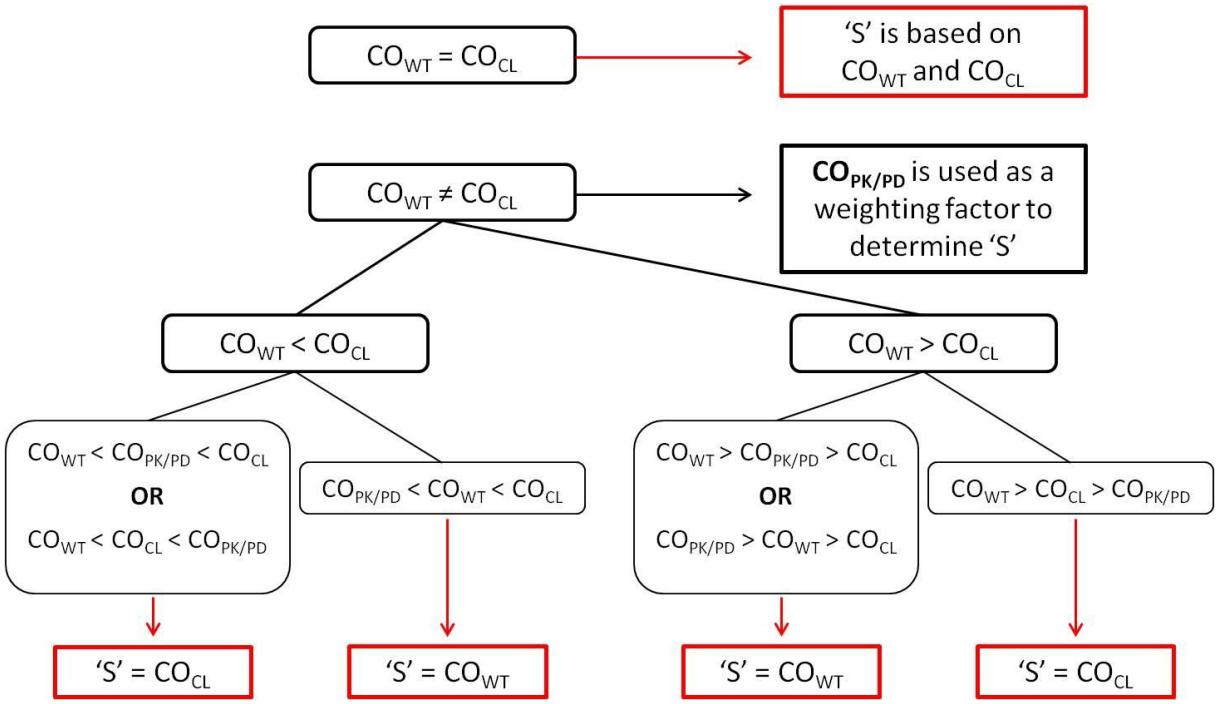

Figure 6. Decision tree to select a breakpoint from the three cut-off values, with CO_{WT}, epidemiological cut-off value; CO_{CL}, clinical cut-off value; $CO_{PK/PD}$, PK/PD cut-off value; S, clinical breakpoint set as susceptible (adapted from Toutain, 2015).

4. Antimicrobial drugs

Antimicrobial drug resistance is a major concern worldwide. Also in the poultry industry, it is of great importance to apply antimicrobial agents prudently, concerning public health and food safety (Agunos et al., 2014). In this respect, fluoroquinolones have already been withdrawn for use in poultry in the United States (FDA, 2005; Nelson et al., 2007). Therefore, the use of antimicrobials which are not registered in human medicine is preferred. Florfenicol and the new generation macrolide GAM are only used in veterinary medicine and are used to treat respiratory infections in swine and cattle, suggesting a possible therapeutic effect against an *O. rhinotracheale* infection in turkeys.

O. rhinotracheale has the ability to invade the respiratory tract of turkey poults. Therefore, an antimicrobial drug with a high distribution to lung tissue is preferred. Florfenicol and GAM may be of interest, due to their interesting PK characteristics. In order to gain insight into the distribution to the respiratory tissue and PK properties of those drugs, the concentration should be determined in the target tissues, and not only in plasma. In turkeys, there are no PK studies of antimicrobials in respiratory tissue performed.

4.1. Florfenicol

4.1.1. Structure and physicochemical properties

Florfenicol is a structural analogue of chloramphenicol (CAP). The latter is obtained from the bacterium *Streptomyces venezuelae*. Since CAP has severe adverse effects in humans, the most important being bone marrow depression, the administration of the drug is only allowed in special cases. In veterinary medicine, the use of CAP is forbidden in food-producing animals (EU 37/2010). Therefore, two structure analogues have been developed, namely thiamphenicol (TAP) and FF. TAP differs from CAP by the replacement of the nitro group by a sulfomethyl group. FF lacks the nitro group as well, but has a fluorine atom at the 3’ carbon position (Fig. 7). This replacement enhances the antibacterial activity, broadens its spectrum, as well as enhancing its bioavailability.

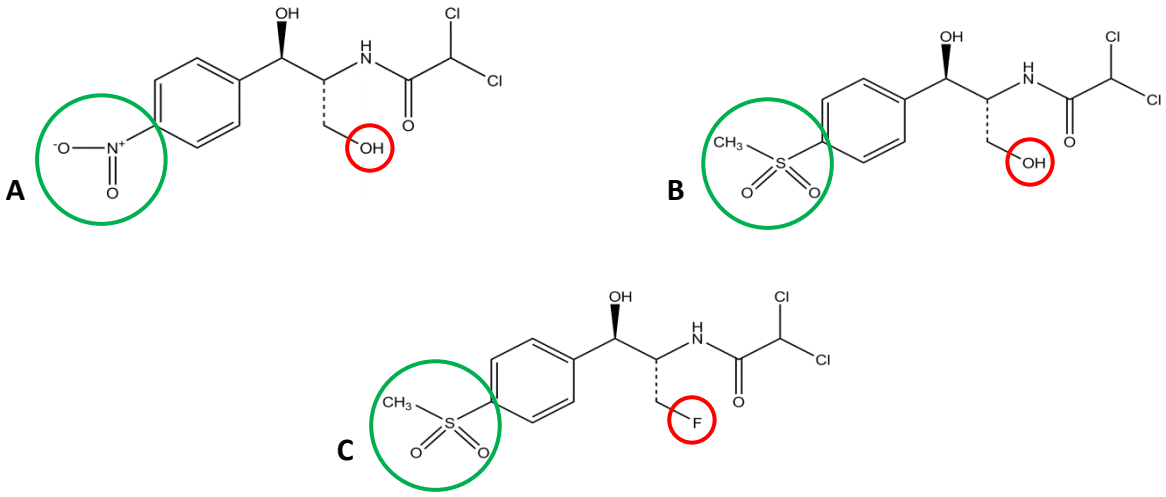

Figure 7. Chemical structures of chloramphenicol (A), thiamphenicol (B) and florfenicol (C). Thiamphenicol and florfenicol have a sulfomethyl group instead of the nitro group (green circle). Florfenicol has a fluorine atom at the 3' carbon position (red circle).

The molecular formula of FF is $C_{12}H_{14}Cl_2FNO_4S$, with a molecular mass of 358.21 g/mol. FF has a low log P value of -0.12, which corresponds with rather hydrophilic molecules, although FF is poorly soluble in aqueous solutions. With a pK_a value of 9.03, FF is unionized in a pH range from 3 to 9. Due to its lipophilicity and unionized part at physiological pH of 7.4, FF shows a good tissue penetration (Schwarz et al., 2004).

4.1.2. Mechanism of action and spectrum

The phenicols inhibit the growth of bacteria by preventing bacterial protein synthesis, in particular the process of transpeptidation at the 23S ribosomal RNA in the 50S subunit of ribosomes. These drugs bind irreversible to different bases of the peptidyl transferase center and prevent protein elongation (Fig. 8) (Dowling, 2013).

Comparable with CAP, FF has a broad spectrum activity with a slightly wider range, including Gram-positive and Gram-negative, aerobic and anaerobic bacteria, chlamydiae, mycoplasmas and rickettsiae. It Is highly active against bacterial pathogens which are involved in respiratory diseases. Approved clinical breakpoints for FF are available for pathogens related with swine (SRD) as well as bovine (BRD) respiratory disease, such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica* and *Mycoplasma hyopneumoniae* for SRD and *P. multocida*, *Mannheimia hemolytica*, *Histophilus*

somni and *M. bovis* for BRD. The MIC clinical breakpoints of the above mentioned bacterial species for susceptibility to FF are all set at 2 µg/mL (CLSI, 2013). FF is also registered for the treatment of pododermatitis in cattle caused by *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* (Schwarz et al., 2004). Furthermore, FF has been approved in Europe as treatment for fish and chickens (EMA, 2002). In turkeys, FF can be applied against respiratory infections with *O. rhinotracheale* (Marien et al., 2007). Also for *Mycoplasma gallisepticum*, the MIC_{50} of FF was low, 1 and 0.5 µg/mL (Gharaibeh and Al-Rashdan, 2011). Enterobacteriaceae are less susceptible, for example the MIC_{50} and MIC_{90} against *Salmonella enterica* isolates was 8 and 16 µg/mL, respectively, and the MIC_{90} for *Salmonella* Dublin was 32 µg/mL (Clemente et al., 2013; Dowling, 2013). Salmon and Watts (2000) reported a MIC_{50} and MIC_{90} for FF against *E. coli* of 4 and 8 µg/mL, respectively. Notwithstanding, Gregova et al. (2012) and Kashoma et al. (2014) found a high susceptibility of FF against *E. coli* and *Campylobacter* spp.

Because there are no clinical breakpoints of FF for avian pathogens, the above-mentioned MIC values have to be critically interpreted. These values depend not only on the pathogen-drug interaction but also on the used population of strains of the pathogen, and the method of determination. Moreover, these values have to be compared with the PK of the antimicrobial and the clinical outcome in field studies.

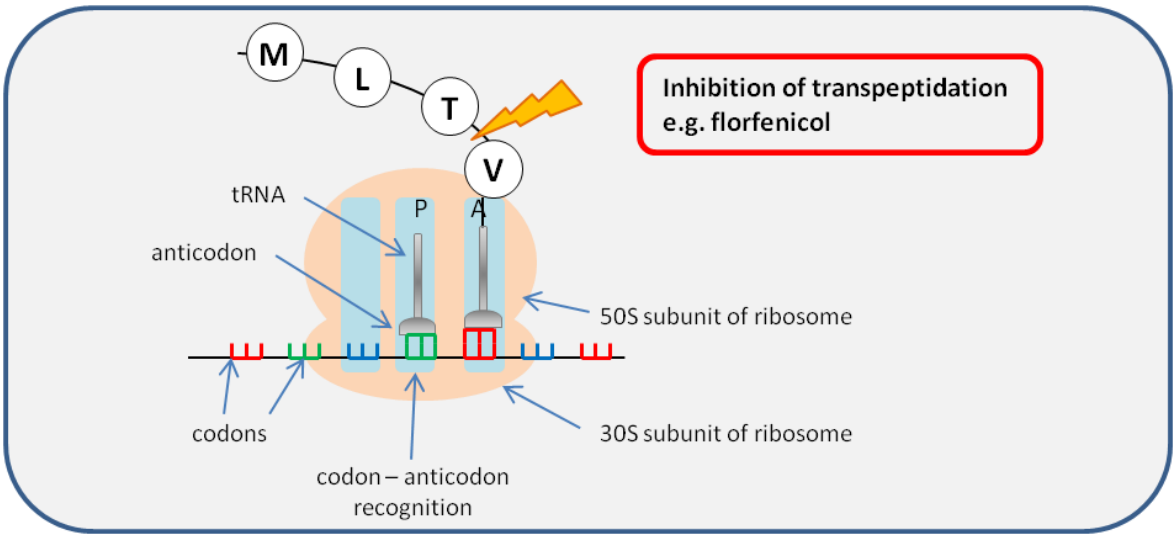

Figure 8. Diagram of the mechanism of action of florfenicol, which inhibits the transpeptidation of the growing peptide chain, during the bacterial protein synthesis (adapted from Rang et al., 2003). This reaction, which occurs between the aminoacyl acceptor (A) site and the peptidyl (P) donor site, is catalysed by the enzyme peptidyl transferase.

4.1.3. Mechanism of resistance

Chloramphenicol acetyltransferases (CATs) are responsible for the enzymatic inactivation of CAP by acetylation of the hydroxyl groups. This results in preventing the drug to bind to the 50S ribosomal subunit. Although CATs are responsible for the first and most frequently encountered mechanism of resistance for CAP, FF doesn't have this mechanism due to the replacement of the hydroxyl group by a fluorine atom. Consequently, CAP-resistant strains, in which resistance is based on the activity of CAT, are still susceptible to FF (Schwarz et al., 2004; Dowling, 2013). The genes coding for CATs or specific transporters are often present on mobile genetic elements, such as plasmids, transposons or gene cassettes (Dowling, 2013).

Another mechanism of resistance is the efflux of phenicols by efflux proteins on the bacterial cell wall. Both CAP and FF can be exported from the bacterial cell by specific transporters or multidrug transporters belonging to the Major Facilitator Superfamily (MFS). Specific transporters, which have a narrow substrate spectrum, lead to higher levels of resistance as compared to the multidrug transporters. The latter are involved in the efflux of a wide range of unrelated substances (Schwarz et al., 2004).

FloR genes are associated with multiresistance gene clusters and are detected in avian pathogenic *E. coli* and *Salmonella enterica* (Keyes et al., 2000; Ahmed et al., 2007; Ahmed et al., 2013).

4.1.4. Toxicity and interactions

The use of CAP has been restricted due to severe adverse effects in humans such as dose-independent irreversible aplastic anemia and dose-dependent bone marrow suppression (Schwarz et al., 2004). Therefore, FF was developed and registered solely for veterinary use. The nitro-group, which was considered to be responsible for the bone marrow suppression, has been replaced by a sulfomethyl group (Fig. 7). Nevertheless, FF might exert less life-threatening side effects, such as transient diarrhea, feed refusal, peri-anal inflammation or rectal eversion. These symptoms were described in cattle and swine and should resolve within a few days of discontinuing treatment (Dowling, 2013).

Phenicols should not be used concurrent with macrolides as they both bind at the 50S ribosomal site and act as competitors. Phenicols have an antagonistic action to fluoroquinolones due to the mode of action of the phenicols. The latter inhibit protein

synthesis, resulting in a deficiency of production of autolysins, which are necessary for cell lysis after fluoroquinolone interference with bacterial DNA. Furthermore, phenicols inhibit microsomal enzyme activity, leading to a delayed hepatic biotransformation. Consequently, the pharmacological effect of drugs that are dependent on these enzymes for their biotransformation, like barbiturates, can be prolonged (Dowling, 2013). Moreover, simultaneous administration of FF and inhibitors of P-glycoprotein or cytochrome P450 enzyme complex (CYP450) 3A may lead to increased AUC values and decreased elimination of FF in rabbits (Liu et al., 2012).

4.1.5. Pharmacokinetic properties

The plasma PK of FF has been described in many animal species, including mammals, birds and fish. Tables 4 and 5 give an overview of the PK parameters in several animal species.

In general, FF is absorbed very rapidly after both IM and oral (PO) administration. For oral administration, it is important to know if the absorption depends on the prandial status of the animal. Jiang et al. (2006) found no significant effect of prior feeding on the bioavailability of the drug in pigs. In birds, the bioavailability after IM and PO administration is generally high (> 75%, El-Banna, 1998; Shen et al., 2003; Switala et al., 2007; Ismail and El-Kattan, 2009; Koc et al., 2009b). One study demonstrated a lower oral bioavailability of 55% in broiler chickens (Afifi and Abo El-Sooud, 1997). This could be attributed to the fed status of the chickens or a partial absorption from the gastro-intestinal tract. In Leghorn chickens and Taiwan native chickens, the C_{max} was much higher and the time to C_{max} (t_{max}) shorter compared to the other avian species.

The plasma protein binding is low, around 20% in chickens as well as in healthy and *Pasteurella* infected ducks (Afifi and Abo El-Sooud, 1997; El-Banna, 1998; Chang et al., 2010). Accordingly, FF distributes well among tissues, such as lung, muscle, bile and kidney (Dowling, 2013), resulting in a moderate volume of distribution (V_d) in avian species and rabbits (≥1 L/kg). However, in sheep, goats and camels the V_d was below 1 L/kg. Liu et al. (2003) described a rapid and extensive penetration into the respiratory tract in pigs infected with *A. pleuropneumoniae*. In mammals as well as in avian species, FF concentrates in kidney, intestine, lung and bile (Adams et al., 1987; Afifi and Abo El-Sooud, 1997; Chang et al., 2010), but no further PK parameters are known.

In chickens and Japanese quails the total body clearance (Cl) is similar, around 1.5 L/kg/h, while it seems to be higher for pigeons and quails, 3.9 and 5.3 L/kg/h, respectively. On the contrary, turkeys have a Cl which is comparable to that of mammals, <0.5 L/kg/h. The half-life of elimination ($t_{1/2el}$) in turkeys, broiler chickens, quails and pigeons was comparable after either intraveneous (IV), IM or PO administration (range from 1.16 to 3.76 h). The elimination of FF was remarkably longer in ducks, Leghorn chickens and Taiwan native chickens, with a $t_{1/2el}$ of 7.42, 10.96 and 10.19 h respectively. Also in mammals, there is a wide range of $t_{1/2el}$. In goats, camels and rabbits this value is similar to turkeys and chickens, whereas pigs and cattle have much longer $t_{1/2el}$. A flip-flop phenomenon can occur as the commercial injectable formulation of FF is long-acting, leading to a prolonged elimination due to the slow absorption from the injection site (IM or SC) (Dowling, 2013).

Florfenicol amine is the most important metabolite. Since its depletion from the liver is very slow, it is used as a marker residue for the determination of maximum residue levels (MRLs) (EU 37/2010; Dowling, 2013). Excretion of FF and its metabolites occurs mainly via urine (EMA, 1999).

As described above, and can be seen in Tables 4 and 5, the PK properties vary enormously between different mammal and avian species. Also Baert and De Backer (2003) demonstrated differences in PK properties of non-steroidal inflammatory drugs between avian species. Rivière et al. (1997) performed an interspecies allometric analysis of the PK of 44 drugs. However, this approach seemed not suitable for CAP. Therefore, it is of great importance to study the PK in the species of interest and not to extrapolate from other animal species. Furthermore, in case of sickness and fever, enhanced extravascular distribution occurs, resulting in reduced circulating plasma concentrations (El-Banna, 1998). Consequently, the PK can differ depending on the health status of the animals.

4.1.6. Pharmacodynamic properties

The main purpose of FF is its antibacterial action against several pathogens which are responsible for respiratory tract infections, urinary and gastrointestinal tract infections. FF is considered to be mainly a time dependent drug, in which the T>MIC in plasma must be at least 40% of a 24 h dosing interval (Hesje et al., 2007).

An excellent response of FF in BRD and SRD can be attributed to the low resistance of cattle and swine pathogens like *M. haemolytica*, *P. multocida*, *A. pleuropneumoniae*, which all have

a MIC_{90} below 1 µg/mL (Shin et al., 2005). T>MIC of FF against *M. haemolytica* and *P. multocida* was longer than 75 h (Sidhu et al., 2014). After an oral bolus administration of 30 mg/kg BW FF to chickens, the plasma concentration remained for 11 h above a therapeutic concentration of 2 µg/mL (Shen et al., 2003).

Salmon and Watts (2000) evaluated the sensitivity of several bacterial pathogens for FF. The authors observed a MIC_{50} and MIC_{90} against *E. coli* of 4 and 8 µg/mL, respectively. Other Gram-negative bacteria showed similar values, except for *Pseudomonas* spp., which have a MIC_{50} as well as a MIC_{90} of >64 µg/mL. The MIC_{90} of Gram-positive organisms, such as *Staphylococci*, *Streptococci* and *Enterococci*, was 4 µg/mL. The authors reported also geographic differences for several antibiotics, but not for FF. In turkeys, FF administered in drinking water at a dose of 20 mg/kg BW was reported a good therapy to cure from an *O. rhinotracheale* infection (Marien et al., 2006). In this study, both the clinical symptoms and the bacterial titres were reduced during and after treatment, but no correlation was made between PK and PD.

Table 4. Plasma pharmacokinetic properties of florfenicol in different avian species.

	ROA	Dose mg/kg BW	AUC_{inf} µg.h/mL	$t_{1/2el}$ h	t_{max} h	C_{max} µg/mL	V_d L/kg	Cl mL/kg/h	F %	References
Broiler chicken	IV	30	19.00	2.89	-	-	6.47	1.61	-	Afifi and Abo El-Sooud, 1997
	IM	30	18.33	3.40	1.67	3.82	ND	ND	97	Afifi and Abo El-Sooud, 1997
	PO	30	10.50	1.78	1.05	3.20	ND	ND	55	Afifi and Abo El-Sooud, 1997
	IV	30	18.00	3.21	-	-	5.33	1.63	-	Ismail and El-Kattan, 2009
	IM	30	17.84	3.24	1.50	4.50	ND	ND	99	Ismail and El-Kattan, 2009
	IV	30	29.45	3.02	-	-	1.15	1.02	-	Shen et al., 2003
	IM	30	28.75	2.38	0.73	6.79	ND	ND	98	Shen et al., 2003
	PO	30	27.59	2.25	1.35	5.82	ND	ND	94	Shen et al., 2003
Leghorn chicken	PO	30	38.49	10.96	0.53	9.42	ND	ND	ND	Chang et al., 2010
Taiwan native chicken	PO	30	26.07	10.19	0.30	10.42	ND	ND	ND	Chang et al., 2010
Duck	IV	30	-	7.17	-	-	5.15	0.61	-	El-Banna, 1998
	IM	30	-	7.42	1.15	2.99	ND	ND	77	El-Banna, 1998
Pigeon	IV	30	7.54	1.82	-	-	5.76	3.88	-	Ismail and El-Kattan, 2009
	IM	30	7.55	2.00	1.50	2.90	ND	ND	100	Ismail and El-Kattan, 2009
Quail	IV	30	5.00	1.24	-	-	4.70	5.30	-	Ismail and El-Kattan, 2009
	IM	30	4.91	1.16	1.50	2.10	ND	ND	97	Ismail and El-Kattan, 2009
Japanese quail	IV	30	23.00	ND	-	-	8.70	1.30	-	Koc et al., 2009b
	IM	30	12.30	ND	ND	ND	ND	ND	79	Koc et al., 2009b
Turkey	IV	30	97.06	2.34	-	-	1.06	0.32	-	Switala et al., 2007
	PO	30	77.62	3.76	2.00	12.25	ND	ND	82	Switala et al., 2007

ROA, route of administration; AUC_{inf}, area under the plasma concentration-time curve from time 0 to infinity; $t_{1/2el}$, half-life of elimination; t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; V_d, volume of distribution; Cl, total body clearance; F, absolute bioavailability.
IV, intravenous; IM, intramuscular; SC, subcutaneous; PO, per oral; ND, not determined; -, not possible to determine due to the ROA

Table 5. Plasma pharmacokinetic properties of florfenicol in different mammal species.

	ROA	Dose mg/kg BW	AUC_{inf} µg.h/mL	$t_{1/2el}$ h	t_{max} h	C_{max} µg/mL	V_d L/kg	Cl mL/kg/h	F %	References
Camel	IV	20	60.61	1.44	-	-	0.89	0.33	-	Ali et al., 2003
	IM	20	41.93	2.52	1.51	0.84	ND	ND	69	Ali et al., 2003
Cattle	SC	40	175.10	27.54	2.96	6.04	ND	0.23*	ND	Sidhu et al., 2014
Goat	IV	20	74.07	1.19	-	-	0.57	0.27	-	Ali et al., 2003
	IM	20	58.73	2.12	1.13	1.21	ND	ND	61	Ali et al., 2003
Pig	IV	20	90.10	6.70	-	-	1.50	0.23	-	Jiang et al., 2006
	IM	20	84.30	17.20	1.00	3.50	ND	ND	97	Jiang et al., 2006
	PO	20	132.10	10.00	1.50	9.90	ND	ND	149	Jiang et al., 2006
Rabbit	IV	25	44.59	1.21	-	-	0.98	0.56	-	Koc et al., 2009a
	IM	25	39.10	1.49	1.56	8.65	ND	ND	89	Koc et al., 2009a
Sheep	IV	30	119.21	18.71	-	-	1.86	0.25	-	Shen et al., 2004
	IM	30	101.95	9.57	1.34	7.01	ND	ND	86	Shen et al., 2004
	IV	20	62.45	1.31	-	-	0.69	0.30	-	Ali et al., 2003
	IM	20	49.56	2.28	1.44	1.04	ND	ND	66	Ali et al., 2003

ROA, route of administration; AUC_{inf}, area under the plasma concentration-time curve from time 0 to infinity; $t_{1/2el}$, half-life of elimination; t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; V_d, volume of distribution; Cl, total body clearance; F, absolute bioavailability.
IV, intravenous; IM, intramuscular; SC, subcutaneous; PO, per oral; ND, not determined; -, not possible to determine due to the ROA
* Cl/F, clearance scaled by bioavailability

4.2. Gamithromycin

4.2.1. Structure and physicochemical properties

Gamithromycin is an antimicrobial agent belonging to the group of macrolides. These antibiotics have three common structural characteristics, a large lactone ring (*macro* meaning large, *olide* meaning lactone), a ketone group and a glycosidically linked amino sugar (Fig. 10) (Martin, 1998; Giguère, 2013). According to the number of atoms in the lactone ring, the macrolides are classified in a 12- (which is no longer used in clinical practice), 13-, 14-, 15- and 16-membered subgroup (Fig. 9).

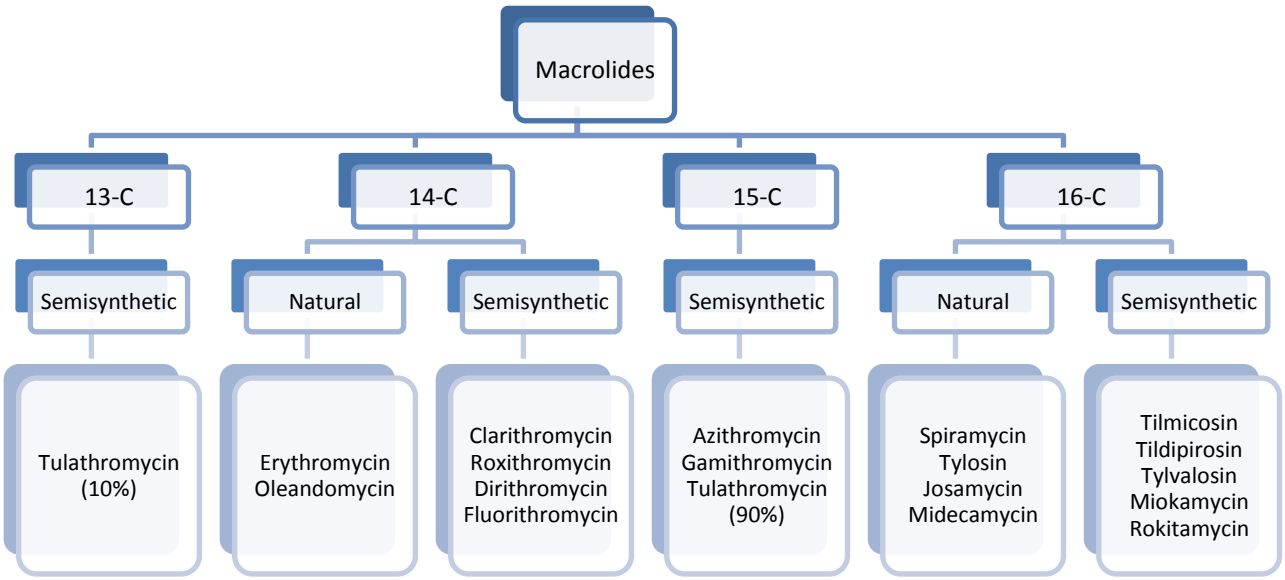

Figure 9. Classification of macrolide antimicrobial agents, according to their nature (natural or semisynthetic) and to the size of the lactone ring (adapted from Giguère, 2013).

Most of the macrolides are produced by *Streptomyces* spp., with erythromycin (Fig. 10) as the prominent molecule, originating from the organism *Streptomyces erythreus*. However, the use of these natural compounds is limited because of their instability in gastric acid and, consequently, poor oral bioavailability. Therefore, semisynthetic derivates have been developed through esterification, salt formation or structural modification (Kirst, 1991; Papich and Rivière, 2009).

The macrolides are weak bases with a dimethylamine group, which results in a pK_a that varies between 6 and 9 (Babić et al., 2007; Papich and Rivière, 2009; Beale, 2011). Older macrolides contain just one functional group with associated pK_a value, such as 8.88 and

7.73 for erythromycin and tylosin, respectively (McFarland et al., 1997). On the other hand, the new generation macrolides, such as azithromycin, tilmicosin and tulathromycin, can be di-basic or even tri-basic (McFarland et al., 1997; Douthwaite et al., 2011; Reeves, 2012).

GAM is a 15-membered erythromycin derivate, with a uniquely positioned alkylated nitrogen at the 7a carbon of the lactone ring, which is typical for azalides (Fig. 10). This structure appears to prevent the gastric degradation, increases the antibacterial activity, improves tissue penetration and prolongs $t_{1/2\ el}$ (Peters et al., 1992; Piscitelli et al., 1992).

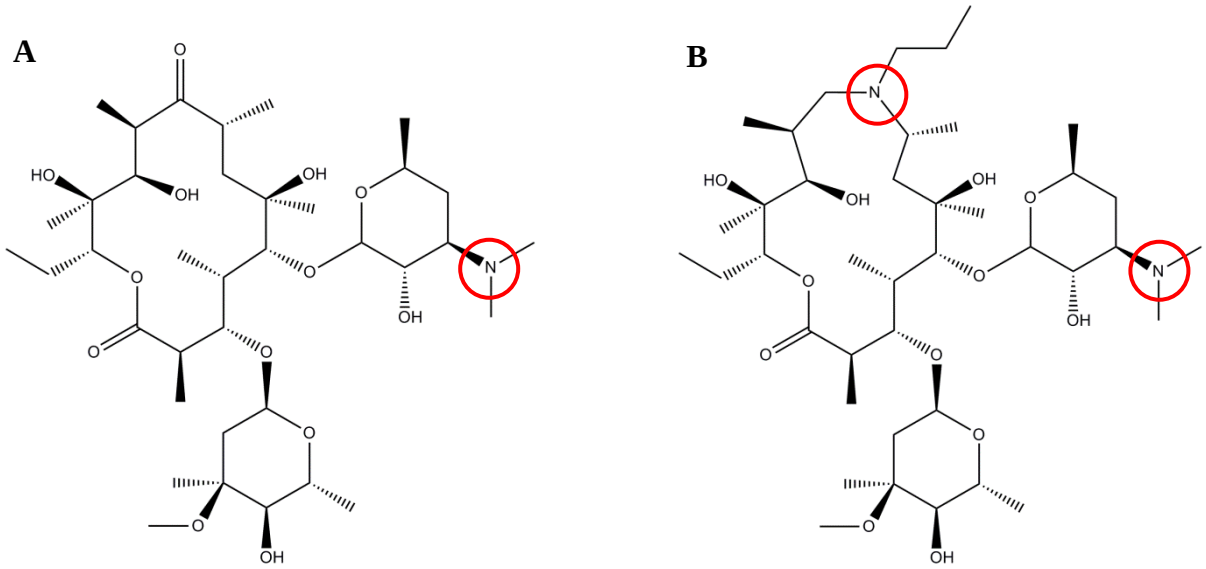

Figure 10. Chemical structures of (A) erythromycin, a 14-membered lactone ring with 2 sugars, clandinose and desosamine, and (B) gamithromycin, a 15-membered lactone ring with the same sugars as erythromycin and additional an alkylated nitrogen at the 7a carbon of the lactone ring. The red circle indicates the groups responsible for the pK_a values: 8.88 for erythromycin, 8.88 and 9.78 for gamithromycin.

The molecular formula of GAM is $C_{40}H_{76}N_2O_{12}$, with a molecular mass of 777.04 g/mol. A log P value of 4.69 indicates that GAM is lipophilic and according to the law of Fick, results in a rapid diffusion through cell membranes. GAM is a dibasic molecule, with two pK_a values, 8.88 and 9.78. The pK_{a1} is situated on the basic dimethylamine group, which is the same for all macrolides (Babić et al., 2007). The alkylated nitrogen, which is specific for GAM, results in a pK_{a2}. In contrast with the neutral form, the ionized form is more water soluble. GAM is completely ionized at pH <7, resulting in ion trapping in macrophages (pH 4.8), where macrolides preferentially concentrate.

4.2.2. Mechanism of action and spectrum

Macrolides have the same mode of action as FF. These compounds bind irreversible to a receptor site on the 50S subunit of the bacterial ribosome, inhibiting the translocation process. Subsequently, they prevent the amino acid transfer to the growing peptide chain and thus inhibit protein formation (Fig. 11) (Cobos-Trigueros et al., 2009).

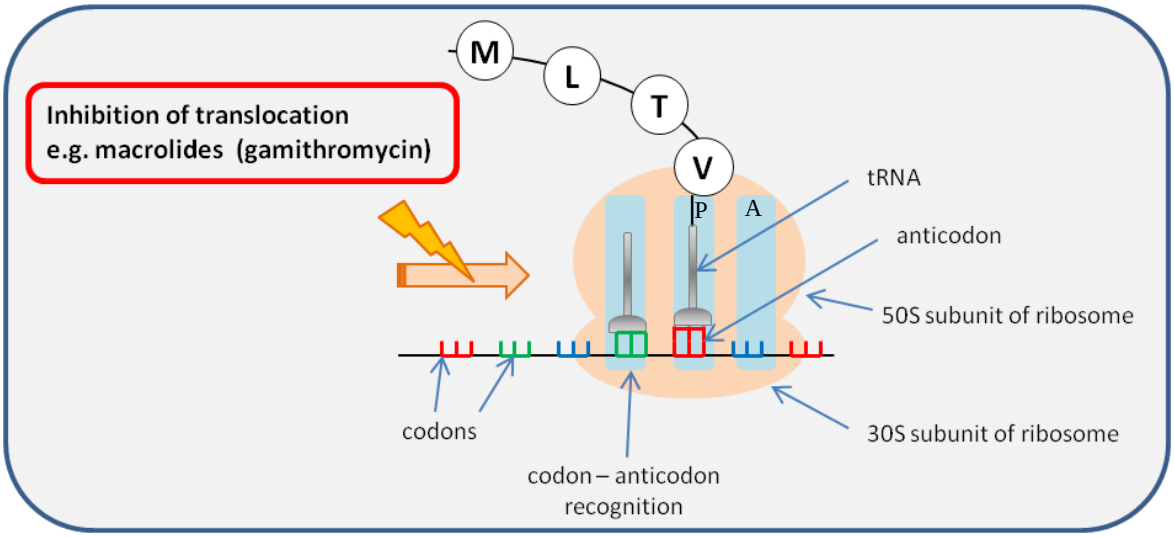

Figure 11. Diagram of the mechanism of action of GAM, which inhibits the translocation from the aminoacyl acceptor (A) site and the peptidyl (P) donor site, during the bacterial protein synthesis (adapted from Rang et al., 2003).

In general, macrolides act bacteriostatic, but at higher doses they can also be bactericidal. It is known that the 14- and 15-membered macrolides have a time-dependent action (Tamaoki, 2004).

The spectrum of activity of the macrolides is mainly against Gram-positive micro-organisms and also against many intracellular bacteria. For humans, clinical indications of macrolides include *Mycoplasma pneumoniae*, infections of the respiratoy tract, skin and soft tissues with *Steptococcus* spp. and *Staphylococcus* spp., diphtheria, gastroenteritis caused by *Campylobacter jejuni* and urethritis/cervicitis caused by *Chlamydophila trachomatis*, *Neisseria gonorrhoeae* or *Uroplasma urelyticum* (Anadon and Reeve-Johnson, 1999). In veterinary medicine, these drugs are used to treat pneumonia and mastitis. In particular, the azalides have a similar spectrum to that observed for erythromycin, but with some differences. Azithromycin is less active against Gram-positive bacteria, although it has more potency against Gram-negative bacteria (Beale, 2011). Also GAM has activity against both

Gram-positive and -negative bacteria, such as *M. haemolytica*, *P. multocida*, *H. somni*, *M. bovis*, *M. mycoides* subspecies mycoides, *Streptococcus equi* subspecies zooepidemicus, *Rhodococcus equi*, *Bacteroides melaninogenicus*, *Treponema* spp. and *Dichelobacter nodosus* (Bagott et al., 2011; Forbes et al., 2011; Sargison and Scott, 2011; Evans et al., 2012; Giguère, 2013; Mitchell et al., 2013, Forbes et al., 2014; Strobel et al., 2014; Hildebrand et al., 2015). At this time, GAM is only registered to treat BRD, but is used off-label against other bacterial infections in sheep and foal (Sargison and Scott, 2011; Hildebrand et al., 2015). Just recently, the manufacturer has intentions to register the product for treatment of SRD and MRLs have been established for porcine species (EMA, 2015).

4.2.3. Mechanism of resistance

Resistance against macrolides can occur through different mechanisms. The first mechanism is the ribosomal target modification due to rRNA methylation. This methylation, most often at the 23S rRNA changes the conformation of the ribosome, preventing the antimicrobial from binding to the bacterial ribosome (Zhanel et al., 2001). Ribosomal methylation results in a high level of resistance and is, in addition, responsible for cross-resistance between the macrolides, lincosamides and streptogramin, the so-called MLS resistance (Zhanel et al., 2001). The mechanism of ribosomal target modification is encoded by the erythromycin-resistant methylase (*erm*) genes. These genes are widely distributed in Gram-positive as well as Gram-negative bacteria and can be located on plasmids or transposons (Giguère, 2013). The expression of the *erm* genes can be inducible or constitutive. The constitutive form, when *erm* mRNA is always active, is related to the MLS resistance. On the other hand, the inducible *erm* genes are synthesized in an inactive form and become active in the presence of inducing macrolides. The latter are 14- and 15-membered macrolides, while lincosamides and 16-membered macrolides are weaker inducers of resistance (Zhanel et al., 2001; Giguère, 2013).

Secondly, the presence of efflux pumps in the cell or cell membrane results in macrolide resistance. There is a wide range of efflux genes (*mef* genes), whereby some only interfere with 14- and 15-members, whereas other genes lead to MLS resistance (Giguère, 2013). These efflux pumps have a role in the acquired resistance, as in some Gram-positive bacteria, as well as in the natural resistance of Gram-negative bacteria such as *Enterobacteriaceae* (Zhanel et al., 2001; Bozdogan and Appelbaum, 2004).

A less common mechanism of resistance is caused by enzymatic inactivation. The inactivating enzymes are esterases and phosphorylases (Giguère, 2013).
Mutation in ribosomal proteins is also a rare cause of macrolide resistance. These mutations appear in the 23S rRNA and/or ribosomal protein genes (Zhanel et al., 2001; Giguère, 2013).
Moreover, a macrolide efflux pump and a macrolide-inactivating phosphotransferase are involved in GAM resistance for several isolates of *M. haemolytica* and *P. multocida* (Michael et al., 2012; Rose et al., 2012; Olsen et al., 2015).

4.2.4. Toxicity and interactions

Macrolides have in general low adverse effects in man and animal species. An important side effect for all macrolides is their irritating nature (irritation can be pharmacological in nature or can be due to the poor solubility of macrolides), which leads to pain at the site of injection (IM, SC), thrombophlebitis and periphlebitis (IV) and inflammation after intramammary administration (Giguère, 2013, Wyns et al., 2015). Erythromycin has dose-related gastrointestinal inconveniences in most animal species, such as nausea, vomiting, diarrhea and intestinal pain. Besides the disruption of the intestinal microbiota, erythromycin binds to motilin receptors resulting in stimulatory effects on smooth muscle. The newer macrolides, like GAM have no serious adverse events, only those mainly associated with the injection, such as pain, swelling and redness (Giguère, 2013; Wyns et al., 2014).

Many macrolides inhibit CYP450 isoenzymes in liver microsomes and enterocytes, especially CYP3A4/5. Hence, macrolides in combination with drugs which depend on CYP450 mediated biotransformation result in an increased concentration of these drugs. For example, ionophoric antibiotics, which have a CYP450 dependent biotransformation, in combination with macrolides (except tylosin, tilmicosin, azithromycin, spiramycin, josamycin and midecamycin) can cause anorexia, depression and myopathy of cardiac and skeletal muscles (Anadon and Reeve-Johnson, 1999). The combination with the ionophoric coccidiostat lasalocid is more safe (Lodge et al., 1988; Islam et al., 2009). Some macrolides (erythromycin, clarithromycin, but not azithromycin) are inhibitors of the intestinal P-glycoprotein (multidrug resistance protein, MDR1), resulting in interactions at the intestinal absorption level. In humans, a prolongation of the QT interval with risk of cardiac arrhythmias has been described when co-administrating macrolides with quinolones (Cascorbi, 2012).

4.2.5. Pharmacokinetic properties

The oral bioavailability of macrolides is low to moderate (30 – 50%). However, salt- or ester-forms show an enhanced oral absorption up to 80% (Zhanel et al., 2001). Despite this incomplete absorption, the low concentration should guarantee a good efficacy of the drug in target tissue (Kowalski et al., 2002). Due to their lipophilic nature, macrolides distribute very well into different tissues, such as the lung, liver, kidney, spleen and the reproductive tract (Anadon and Reeve-Johnson, 1999). Consequently, these PK properties correspond with a two-compartment model and drug plasma concentrations are consistently lower than at the site of action. The $t_{1/2el}$ varies among different macrolides. For instance, in chickens the $t_{1/2el}$ of erythromycin and tylosin after PO administration is short (4.1 h and 2.07 h, respectively) compared to tilmicosin (45 h) (Kowalski et al., 2002; Goudah et al., 2004; Abu-Basha et al., 2007).

Only a few studies reported PK properties of GAM in plasma, lung tissue and PELF of different animal species (Table 6). GAM is fully and rapidly absorbed into the systemic circulation after SC or IM administration, with a C_{max} that shows discrepancy between species. After the administration of the same dose, foals and pigs have much lower plasma concentrations compared to cattle and chickens. In cattle as well as in pigs and chickens, a complete bioavailability was demonstrated after SC administration. The plasma Cl varies among species, with increasing Cl from cattle towards pigs and chicken (Table 6). As stated above, smaller species will eliminate drugs more rapidly compared to large animals. While the V_d remains constant, this results in a decreased $t_{1/2el}$ (Huang et al., 2010; Berghaus et al., 2011; Watteyn et al., 2013a; Wyns et al., 2014). The low plasma protein binding, namely 26.0%, 23.1%, 21.8% and 21.5% in cattle, swine, rat and dog plasma, respectively, is also responsible for the very high V_d (> 20 L/kg) (EMA, 2008b). As already mentioned in general, plasma is not the most accurate matrix to predict the *in vivo* efficacy of macrolides. Therefore, studies of the PK of GAM in the target tissue are even more important. In comparison with plasma, the concentrations in lung tissue and PELF are much higher, due to the accumulation of GAM in the respiratory tract. These high concentrations, combined with the slow elimination of the drug from the target site results in sustained drug concentrations for days following a single injection (Berghaus et al., 2011; Giguère et al., 2011).

GAM is mainly metabolized in the liver, with declad (loss of a cladinose) and M2 (N-dealkylated-declad) as the major residues. The biotransformation of GAM was studied in rats and dogs and was found to be similar in those species (EMA, 2008b). GAM and its metabolites were found to be primarily eliminated in the faeces and secondarily in urine, around 50% and 15%, respectively (EMA, 2008b).

4.2.6. Pharmacodynamic properties

Since macrolides are classified as time-dependent antimicrobial agents with significant post-antibiotic effect, the efficacy of these antibiotics is generally accepted to correlate with both T>MIC and AUC_{last}/MIC (Van Bambeke and Tulkens, 2001; Andes et al., 2004; Hesje et al., 2007; Barbour et al., 2010; Giguère and Tessman, 2011). The MIC_{90} values for *M. haemolytica*, *P. multocida* and *H. somni* are 0.5 µg/mL, 1 µg/mL and 1 µg/mL, respectively (EMA, 2008b). These *in vitro* results have been confirmed in *in vivo* studies in cattle suffering from BRD (Baggott et al., 2011; Torres et al., 2013). Berghaus et al. (2011) evaluated the efficacy of GAM against pneumonia in foals, and established its high activity (MIC_{90} 0.125 µg/mL and 1.0 µg/mL for *S. zooepidemicus* and *R. equi*, respectively). Also *in vivo*, GAM demonstrated good efficacy in the treatment of foals with bronchopneumonia (Hildebrand et al., 2015). In sheep, GAM has been evaluated to control footrot, infected with *D. nodosus*, resulting in good clinical cure rates (Forbes et al., 2014; Strobel et al., 2014). Furthermore, in pigs GAM has a low MIC for *M. hyopneumoniae* (0.25 µg/mL), whereas the MIC for *A. pleuropneumoniae* is higher (2 µg/mL) (Wyns et al., 2014). To the author's knowledge, no data from *in vivo* infection studies in pigs are available yet. At present, also PD studies of GAM in poultry are lacking.

Besides the antimicrobial activities, several reports suggest that macrolides have immunomodulatory properties as well (Kanoh and Rubin, 2010). This immunopharmacology can be described as the influence on the immune system by modifying the endogenous immune responses to the benefit of the host in the treatment of diseases (Hadden and Kishimoto, 1993). Kovaleva et al. (2012) showed that macrolides can temper the inflammatory response at different levels (cytokines, inflammatory cells and structural cells). They considered several *in vitro* and *in vivo* studies with different macrolide antibiotics and pathogens. Notwithstanding, no inhibiting effect of GAM on prostaglandin E2 (PGE2), tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) could be noticed in pigs and calves in a

lipopolysaccharide (LPS) inflammation model (Wyns et al., 2015b; Plessers et al., 2015a). Also induction of neutrophil apoptosis could be induced by macrolides (Chin et al., 1998; Lee et al., 2004; Fisher et al., 2011). The pro-apoptotic effects of tulathromycin are caspase-3 dependent and would alter in function of concentration of the antimicrobial and time of exposure (Fischer et al., 2011). To the authors' knowledge, no studies about the immunomodulatory properties of macrolides in poultry have been reported.

Another PD property of macrolides is the ability to stimulate the gastro-duodenal activity (Giguère, 2013). This pro-kinetic action is based on the activation of the motilin receptor and this at a dose which is lower than the antimicrobial dose (Peeters et al., 1989). No research has been performed yet to confirm the pro-kinetic assets of GAM.

Table 6. Pharmacokinetic properties of gamithromycin in plasma, lung tissue and pulmonary epithelial lining fluid (PELF) in different animal species.

	ROA	Dose mg/kg BW	AUC_{inf} µg.h/mL	$t_{1/2el}$ h	t_{max} h	C_{max} µg/mL	V_d L/kg	Cl mL/kg/h	F %	References
PLASMA										
Cattle	IV	3	4.28	44.90	-	-	24.90	0.71	-	Huang et al., 2010
	SC	6	9.42	50.80	1.00	0.75	ND	ND	110	
Chicken	IV	6	4.00	14.12	-	-	20.89	1.77	-	Watteyn et al., 2013a
	SC	6	4.09	11.63	0.13	0.89	ND	ND	102	
Foal	IM	6	3.96	39.10	1.00	0.33	ND	ND	ND	Berghaus et al., 2011
Pig	IV	6	3.67	16.03	-	-	31.03	1.69	-	Wyns et al., 2014
	SC	6	4.31	18.76	0.63	0.41	ND	ND	118	
LUNG										
Cattle	SC	6	2235.00	93.00	12.00	27.80	-	-	-	Giguère et al., 2011
PELF										
Cattle	SC	6	348.00	50.60	24.00	4.61	-	-	-	Giguère et al., 2011
Foal	IM	6	117.00	63.60	24.00	2.15	-	-	-	Berghaus et al., 2011

ROA, route of administration; AUC_{inf}, area under the plasma concentration-time curve from time 0 to infinity; $t_{1/2el}$, half-life of elimination; t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; V_d, volume of distribution; Cl, total body clearance; F, absolute bioavailability.
IV, intravenous; IM, intramuscular; SC, subcutaneous; ND, not determined; -, not possible to determine due to the ROA or the matrix (lung or PELF)

SCIENTIFIC AIMS

Ornithobacterium rhinotracheale is a common avian respiratory pathogen and often affects turkeys during the production round, resulting in significant economic losses. Antimicrobial therapy can be applied in the course of outbreaks, but a careful evaluation has to be made on the choice of antimicrobial agent with respect to antimicrobial resistance and clinically efficacy.

Florfenicol (FF) has been registered to administer in drinking water for many animal species to treat respiratory infections. Besides, gamithromycin (GAM), a newer macrolide, is also used for treatment of respiratory infections and has remarkable pharmacokinetic and pharmacodynamic (PK/PD) properties, such as a high distribution to tissue and a prolonged action after a single bolus administration. Both antimicrobials are registered for veterinary species, but not for turkeys. Since a proper therapeutic effect relies on the understanding of the PK as well as the PD, it is important to obtain these data in the species of interest. Moreover, it is known that FF and GAM have a high distribution to the respiratory tract in several species. But to what extent the concentrations in the lungs and the pulmonary epithelial lining fluid (PELF) differ from those in plasma is still unknown in turkeys.

Although parenteral administration is sometimes used, oral drinking water medication is the most commonly used route of drug administration in intensively reared poultry. Drug intake between animals can vary due to both animal factors and environmental factors. Hence, gaining an insight into the importance of these factors is mandatory for the establishment of an efficient treatment protocol.

Therefore, the **GENERAL AIM** of this doctoral thesis was to evaluate the efficacy of FF and GAM, which are currently not used in poultry, against an *O. rhinotracheale* infection in turkeys, based on a PK/PD approach.

To establish the general aim, following **SPECIFIC AIMS** were formulated**:**

1. To study the pharmacokinetic properties of FF in plasma and respiratory tissue in turkeys, and to relate these with pharmacodynamic characteristics, with respect to different photoperiods and feeding schemes.
2. To investigate the pharmacokinetic properties of the macrolide GAM in plasma and respiratory tissue in turkeys, and to relate these with pharmacodynamic characteristics.

3. To determine the efficacy of FF to treat turkeys infected with *O. rhinotracheale* by means of drinking water medication. Furthermore, to determine the influence of housing conditions, especially photoperiod, on the water intake, and hence to the efficacy of the drug.
4. To evaluate the efficacy of a single bolus administration, either subcutaneous or oral, of GAM as a treatment of *O. rhinotracheale* infection.

EXPERIMENTAL STUDIES

Chapter 1

Pharmacokinetics and pharmacodynamics of selected antimicrobials in turkeys

Chapter 1.1

Pharmacokinetics and pharmacodynamics of florfenicol in plasma, lung tissue and pulmonary epithelial lining fluid in turkeys

Adapted from:

Watteyn A.[*], Russo E.[*], Garmyn A., De Baere S., Pasmans F., Martel A., Haesebrouck F., Montesissa C., De Backer P. and Croubels S. (2013). Clinical efficacy of florfenicol administered in the drinking water against *Ornithobacterium rhinotracheale* in turkeys housed at different environmental conditions: a PK/PD approach. *Avian Pathology*, 42(5), 474-481. [*] Shared first authorship

Watteyn A., Devreese M., De Baere S., De Backer P. and Croubels S. Pharmacokinetics of florfenicol in turkey plasma, lung tissue and pulmonary epithelial lining fluid after continuous or single bolus administration. In preparation.

Abstract

Florfenicol (FF) is registered as a treatment for bovine and swine respiratory diseases. Also turkeys often suffer from respiratory tract infections, although there is no registered formulation based on FF for poultry on the market in Europe. The aim of this study was to evaluate whether FF might be suited for treatment of *Ornithobacterium rhinotracheale* infections in turkeys. First, the minimum inhibitory concentration (MIC) of FF against 38 isolates of the respiratory pathogen *O. rhinotracheale* was determined. Both the MIC_{50} and MIC_{90} were set at 1 µg/mL.

As FF has to be effective at the respiratory tract, data about the antimicrobial concentrations at the target site are needed. Therefore, the concentration and pharmacokinetic characteristics of FF in plasma, lung and pulmonary epithelial lung fluid (PELF) in turkeys were determined, either during and after continuous drinking water medication (30 mg/kg body weight/day for 5 days) or after a single oral bolus (30 mg/kg body weight). Plasma, lung tissue and PELF samples were collected at different time points after administration and FF was quantified by liquid chromatography followed by tandem mass spectometry. After single bolus administration, FF was rapidly absorbed in plasma (mean t_{max} = 1.02 h) and distributed to the respiratory tract (mean t_{max} = 1.0 h). The mean $t_{1/2el}$ in plasma and lung tissue was similar, around 6 h, whereas it was slightly higher in PELF, namely 8.7 h. After oral bolus dosing, the mean C_{max} in plasma was twice as high as in the lung tissue, 4.26 µg/mL and 2.64 µg/g respectively, while in PELF it was much lower, 0.39 µg/mL. Consequently, the time above the MIC_{90} was 67.4% and 50.0% of a 24 h interval in plasma and lung tissue, respectively, but PELF concentrations never exceeded the MIC_{90}. During drinking water medication, lung concentrations were slightly higher than plasma concentrations, although these concentrations were never above the MIC_{90}. FF was not detected in PELF during drinking water medication.

In poultry rearing, drinking water is a commonly used route to administer medication, but drug uptake can be affected by many factors. Therefore, the influence of two important environmental parameters, namely photoperiod and feeding schemes, on FF uptake in turkeys was also evaluated. This experiment was conducted during a 5-day treatment of 30 mg/kg body weight FF administered *via* drinking water and considering different photoperiods and feeding schemes (group 20/4L: photoperiod of 20 h, fed *ad libitum*; group

16/8L: photoperiod of 16 h, fed *ad libitum*; group 16/8R: photoperiod of 16 h, fed *ad libitum* but feed was withdrawn during the dark period and replaced 1 h after lighting). At day 1 of the treatment, plasma concentrations of all groups were above the MIC_{90} during 37.7%, 63.5% and 53.1% of a 24 h interval for respectively 20/4L, 16/8L and 16/8R. Only in the 16/8L and 16/8R groups, the MIC_{90} was also exceeded on day 5 (47.9% and 21.5% of a 24 h interval, respectively). The results demonstrated an important influence of the photoperiod on the pharmacokinetics of FF. It can be advised that the photoperiod should be less than 20 h to have sufficient drug intake. On the other hand, there was no effect of feed restriction on the pharmacokinetics during continuous administration.

Introduction

Florfenicol (FF) has a broad antibacterial action against several pathogens responsible for respiratory tract infections, urinary tract infections and infections of the gastrointestinal system. An excellent clinical response of FF in bovine and swine respiratory diseases can be attributed to the remarkable pharmacokinetic (PK) characteristics and the low resistance of cattle and swine pathogens like *Mannheimia haemolitica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, which all show a minimum inhibitory concentration (MIC_{90}) below 1 µg/mL (Shin et al., 2005). In turkeys, FF has been proven to be effective against *Ornithobacterium rhinotracheale* infection, using drinking water medication during five days at a dose of 30 mg/kg body weight (BW) per day (Marien et al., 2007). However, pharmacodynamic data, such as the minimum inhibitory concentration (MIC), of FF against *O. rhinotracheale* are still lacking. Also, only few data about the PK behaviour of FF in respiratory tissues are available, namely in broiler chickens and pigs (Afifi and Abo el-Sooud, 1997; Li et al. 2002). Due to species dependent differences in anatomy and physiology, PK studies have to be performed in the species of interest. To the authors' knowledge, only one PK study of FF has been performed in turkeys after single bolus administration of 30 mg/kg BW either *per oral* (PO) or intravenously (IV) (Switala et al., 2007), but no concentrations in lung tissue nor pulmonary epithelial lining fluid (PELF), the sites of action, have been reported yet.

Furthermore, medicated drinking water is the most applied route of drug administration to poultry. Besides many advantages, this way of administration has also disadvantages. Drug intake between animals can vary dramatically due to both animal factors (hierarchy, flock size, sex, age, body weight, species, breed, health status, etc.) and environmental factors (temperature, humidity, feed and water availability, photoperiod, etc.) (Vermeulen et al., 2002). Moreover, the solubility and stability of the drug is of utmost importance and may be influenced by many factors of the water quality (such as pH, hardness, contamination). Especially for FF, information about its stability in drinking water is scarce (Hayes et al., 2003). Besides drug intake variability, there can be differences in PK properties of FF after oral administration in fasted and fed broiler chickens (Shen et al., 2003; Baert and De Backer, 2006). These authors reported differences in bioavailability, maximum plasma concentration and time to maximum plasma concentration. Moreover, another study showed the influence

of the applied photoperiod on the PK of doxycycline during drinking water administration in turkeys (Santos et al., 1997). The eating and drinking patterns may alter depending the light scheme (Classen et al., 1994), which could have a huge influence on the uptake of drinking water medication. As FF is a time-dependent antibiotic (Hesje et al., 2007), it is important to have a frequent drug intake. Accordingly, a study with different housing conditions based on photoperiod and feeding schemes is mandatory for the establishment of an efficient treatment protocol.

The first aim of this research was to determine the MIC of FF for *O. rhinotracheale*. Subsequently, the concentrations and PK characteristics in plasma, lung tissue and PELF after single oral bolus administration (30 mg FF/kg BW) were determined. In order to represent field conditions, plasma, lung tissue and PELF concentrations and PK characteristics of FF were also studied during and after continuous drinking water medication during 5 days at the same dose of 30 mg/kg BW/day. The last objective was to evaluate the plasma concentration-time curves, obtained during and after the continuous administration via the drinking water (30 mg/kg body weight, BW) during a 5-day period, taking into account different photoperiods and feeding schemes.

Materials and methods

Veterinary drug, chemicals, solutions and materials

Florfenicol, 2,2-dichloro-N-[1S,2R)-1-(fluoromethyl)-2-hydroxy-2-[4-(methylsulfonyl)-phenyl]ethyl]-acetamide, used for the animal experiments was obtained from Zhejiang Hisoar Pharmaceutical Co., LTD (Zhejiang, China). Because of the low water solubility (1 mg/mL), the FF bolus for the single oral bolus PK study, was given as a suspension of FF in tap water at a concentration of 6 mg/mL. For the continuous drinking water PK study, the medicated drinking water was prepared daily by stirring an appropriate solution (mean ± SD concentration was 74.3 ± 3.4 mg FF/L tap water) for 30 minutes, followed by sonication for 20 minutes to dissolve the FF.

FF standard (99.0% purity) used for the analytical experiments was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and the internal standard (IS) thiamphenicol (TAP, >97.5% purity) was purchased from Sigma-Aldrich (Bornem, Belgium).

All products (sodium hydroxide and acetic acid) and reagents (high-performance liquid chromatography (HPLC) grade methanol and water, analytical grade ethyl acetate) were purchased from VWR (Leuven, Belgium). Ultra-high performance liquid chromatography (UHPLC) water and acetonitrile (ACN) were obtained from Biosolve (Valkenswaard, The Netherlands).

Millex-GV PVDF filter units (0.22 µm) were obtained from Millipore (Brussels, Belgium).

Stock solutions (1 mg/mL) of each analyte were prepared in methanol. By diluting the stock solutions with methanol, working solutions of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 400 µg/mL of FF and of 50 µg/mL of TAP were obtained. The FF stock and working solutions were stable for 9 months at 2-8 °C and TAP solutions were stable for 111 days at 2-8 °C.

Minimum Inhibitory Concentration

The MIC of FF was determined using the agar dilution method. General procedures, weighing and inoculation were according to the Clinical and Laboratory Standard Institute (CLSI) standards. Since no standard conditions for susceptibility testing of *O. rhinotracheale* are described (CLSI, 2013), Mueller Hinton broth supplemented with 5% horse blood was used, as described by Devriese et al. (2001). The plates were incubated for 48 h at 35 °C in a 5% CO_2 atmosphere. Thirty-eight isolates (37 field isolates, originating from poultry, and the *O. rhinotracheale* type strain LMG 9086^T, originally isolated from a turkey) were used. The concentrations of FF tested ranged between 0.016 and 32 µg/mL. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as control strains, as indicated by the CLSI guidelines (CLSI, 2013).

The determination of the MIC was performed in duplo, with an interval of 1 week.

PK-experiment – single oral bolus administration

This study was performed using fifty-four 6-week-old female turkey poults (Hybrid Converter, local commercial turkey farm) with a mean (± SD) BW of 2.063 (± 0.195) kg (Hybrid Converter, local commercial turkey farm) and were housed according to the requirements of the European Union (Anonymous, 2010). The light scheme was set at 16 h light and 8 h dark. After a fasting period of 12 h, the birds received a FF bolus of 30 mg/kg BW by gavage in the crop, followed by rinsing with tap water. Four hours after the bolus administration, the birds received feed.

Blood (1 mL) was collected from six turkeys by venipuncture from the medial metatarsal vein into heparinised tubes (Vacutest Kima, Novolab, Geraardsbergen, Belgium) at different time points, before (time 0) and post administration (p.a.; 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10 and 24 h) for PK analysis. Plasma was separated by centrifugation and stored at ≤ -15 °C, pending analysis.

Furthermore, animals (n=6) were sacrificed at different time points to collect plasma, lung tissue and PELF. Euthanasia was performed at 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after the oral bolus administration. Birds were anesthetized by an intramuscular injection of a combination of xylazine (Xyl-M 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100, Virbac, Wavre, Belgium), followed by exsanguination. The whole right lung was removed for FF analysis. The complete left lung was used to collect PELF as described by Bottje et al. (1999). In brief, after weighing the lung, it was lavaged with heparin-saline (200 units heparin per mL of 0.9% saline) at a volume of 2 mL/g lung through a cannula in the first bronchus. The PELF/saline solution was collected in a petri dish and the amount of fluid was measured to determine the recovery, which ranged from 80.0 to 100%. The fluid was centrifuged (5250 *x g* for 3 min) to remove red blood cells. Both the lung tissue and PELF were stored at ≤ -15 °C until analysis.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2014/68).

PK-experiment – continuous drinking water administration

During an acclimatisation period of five days, water consumption (non-medicated tap water) of twenty 3-week-old female turkey poults (Hybrid Converter, local commercial turkey farm) with a mean (± SD) BW of 0.812 (± 0.074) kg was measured to calculate a correct dose of the medicated drinking water. Thereafter, the turkeys received FF via the drinking water during a 5-day period (target dose: 30 mg/kg BW/day). During the whole experiment, the light scheme was set at 16 h light and 8 h dark.

Blood (1 mL) of six turkeys was collected and stored at the same way as the oral bolus study, except for sampling points. Blood was taken immediately before (time 0), and at 10, 24, 34, 48, 58, 72, 82, 96, 106 and 120 h after the start of the medicated water administration. Also the collection of lung tissue and PELF was similar as the oral bolus experiment, although the euthanasia of four birds at each time point took place at day 2 (24 h), 4 (72 h), 6 (120 h), 8

(168 h) and 10 (216 h), after the start of the continuous drinking water medication. The recovery of the PELF/saline solution ranged between 71.4 to 96.0%.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2013/108).

PK-experiment – different housing and feeding conditions

Eighteen 3-week-old female turkeys, with a mean (± SD) BW of 0.573 ± 0.052 kg were randomly divided in three groups (6 animals/group) with different environmental conditions. The light schedule of the first group was 20 h light (between 8 h a.m. and 4 h a.m.)/4 h dark and they were fed *ad libitum* (20/4L). The light schedule of the second group was 16 h light (between 8 h a.m. and 12 h p.m.)/8 h dark and were fed fed *ad libitum* (16/8L) and the third group was provided the same light cycle and was fed *ad libitum* except during the dark period in which feed was withdrawn (16/8R). These animals received feed again at 1 h after the light was put on.

FF was administered continuously to the three groups *via* the drinking water during a 5-day period (target dose: 30 mg/kg BW). In order to determine the inclusion rate of the drug in the drinking water and to evaluate the real amount of drug ingested, all animals were weighed before the treatment and the water uptake was measured daily from 3 days before until the end of the treatment.

Blood (1 mL) was collected as described above at different time points: immediately before (time 0), at 1, 2.5, 5, 7.5, 10, 15 and 24 h on day 1 and day 5, and on day 6 also at 2, 4 and 8 h. Plasma was separated by centrifugation and stored at ≤-15 °C, pending analysis.

Water samples of the medicated drinking water were collected daily immediately after its preparation and after 24 h, in order to evaluate the homogeneity and stability, respectively. Medicated drinking water was replaced every 24 h by a freshly prepared solution. The medicated drinking water samples were stored at -20 °C pending analysis.

The trial was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2011/027 and EC 2011/096).

Florfenicol analyses in plasma, lung tissue, PELF and drinking water

Quantification of FF in the plasma samples was performed using an in-house developed and validated LC-MS/MS method. The plasma samples (250 µL) were spiked with 12.5 µL of the

IS TAP (50 µg/mL), followed by vortexing (15 s) and an equilibration period (5 min). Subsequent to the addition of 100 µL of sodium hydroxide 1 M, the samples were vortexed (15 s), mixed with 4 mL of ethyl acetate, and again vortexed (15 s). The samples were extracted by horizontal rolling for 20 min, followed by centrifugation (3725 *g*, 10 min). The supernatant was transferred to a glass tube and evaporated to dryness using a nitrogen stream (40 °C). The residue was redissolved into 250 µL of 0.1% acetic acid in water and ACN (80:20, v/v), filtered through a 0.22 µm Millex-GV PVDF filter and transferred to an autosampler vial.

Each lung sample was homogenized with an equal weight of water, using an Ultra Turrax mixer (Ika, Staufen, Germany). A 0.5 g aliquot of this lung tissue homogenate (corresponding with 0.25 g of lung tissue) was used for the FF analysis. Further sample preparation of lung tissue and PELF samples was similar to the plasma samples.

The water samples were diluted 500 times with HPLC water. An aliquot of 250 µL was spiked with 12.5 µL of the IS TAP working solution (50 µg/mL), followed by vortex mixing (15 s) and transfer to an autosampler vial.

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler with temperature controlled tray and column oven, type Surveyor Autosampler Plus, from Thermo Scientific (Breda, the Netherlands).

The chromatographic separation was achieved on a Hypersil Gold column (50 x 2.1 mm internal diameter (i.d.); particle size (d.p.): 1.9 µm) with a guard column of the same type (Hypersil Gold, 10 x 2.1 mm i.d., d.p.: 3 µm), both from Thermo Scientific.

The column temperature was maintained at 45 °C. The injection volume was 10 µL and the analysis was carried out with gradient elution using (A) 0.1% acetic acid in UHPLC water and (B) UHPLC ACN as the mobile phases at a flow rate of 0.30 mL/min. The gradient conditions were as follows: 0 – 2.2 min: 85% A, 15% B; 2.2 - 2.5 min: linear gradient to 20% A; 2.5 – 3.8 min: 20% A, 80% B; 3.8 – 4.0 min: linear gradient to 85% A; 4.0 – 6.0 min: 85% A, 15% B.

The LC column effluent was interfaced to a TSQ Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionisation (h-ESI) probe (all from Thermo Scientific). The analysis of FF and TAP was performed in negative ionization mode.

Instrument parameters were optimised for the analytes. For each compound, the two most intense precursor ion > product ions transitions were selected and monitored in the selected reaction monitoring (SRM) mode. The most intense product ion was used for quantification (i.e. FF: *m/z* 356.1 > 336.0, TAP: *m/z* 354.1 > 185.0).

Prior to routine application, the method was validated in-house by a set of parameters (linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), selectivity) that were in compliance with the recommendations as defined by the European Community (Anonymous, 2002) and with reference guidelines defined in other EU documents (Knecht and Stork, 1974; Heitzman, 1994; VICH GL 49, 2015). Quadratic calibration curves were constructed using matrix-matched calibrator samples (concentration range: 10 – 5000 ng/mL) and the correlation coefficients (r = 0.9983, 0.9998, 0.9981 for plasma, lung tissue and PELF, respectively) and goodness-of-fit coefficients (g = 13.25%, 7.54%, 9.17% for plasma, lung tissue and PELF, respectively) fell within the accepted ranges, i.e. $r \geq 0.99$ and $g < 20\%$, respectively.

Within-run precision (repeatability) and accuracy were determined by analyzing blank samples that were spiked on the same day. The samples were spiked at 25, 250 and 2500 ng/mL; 25, 250 and 2500 ng/g; 25, 100 and 1000 ng/mL, for respectively plasma, lung tissue and PELF samples. The between-run precision and accuracy were determined by analyzing quality control samples together with each analytical batch of samples, run on different days. The concentration levels for plasma, lung tissue and PELF were 100, 250 and 2500 ng/mL; 25, 250 and 2500 ng/g; 100 and 1000 ng/mL, respectively. The results of the validation are shown in Table 1.

As can be seen, these results fell within the accepted ranges for accuracy (-20% to +10% of the theoretical concentration) and precision (within-run precision: relative standard deviation (RSD) ≤ RSD_{max} with RSD_{max} of 15% for concentration levels ≥ 10 and < 100 ng/mL or ng/g, and RSD_{max} of 10% for concentration levels ≥ 100 ng/mL or ng/g; between-run precision: RSD ≤ RSD_{max} with $RSD_{max} = 2^{(1-0.5\log Conc)}$, i.e. 27.9%, 22.6%, 19.7%, 16.0% and 13.9% at 25, 100, 250, 1000 and 2500 ng/mL or ng/g, respectively).

Table 1. Within-run and between-run validation results for FF analyses in plasma, lung tissue and PELF.

	Plasma ng/mL			Lung tissue ng/g			PELF ng/mL		
Within-run	**25**	**250**	**2500**	**25**	**250**	**2500**	**25**	**100**	**1000**
Accuracy (%)	-11.4	5.8	5.3	5.5	-19.8	4.3	-13.7	-2.2	-10.9
Precision (RSD) (%)	9.1	9.9	3.8	6.4	4.2	8.6	11.0	5.2	5.2
Between-run	**100**	**250**	**2500**	**25**	**250**	**2500**	**25**	**100**	**1000**
Accuracy (%)	-8.0	-13.0	-3.8	0.8	3.2	-6.1	ND	-1.7	-2.6
Precision (RSD) (%)	7.3	3.0	10.6	15.2	14.0	12.0	ND	9.6	9.4

ND, not determined

The LOQ was 25 ng/mL for plasma, 25 ng/g for lung tissue and 20 ng/mL for PELF. Values below the LOQ were not included in the plasma concentration-time curves and the PK analysis.

Pharmacokinetic, pharmacodynamic and statistical analyses

Following plasma PK parameters were determined by one-compartmental analysis (WinNonlin 6.3, Pharsight, CA, USA): area under the plasma concentration-time curve from time 0 to infinity (AUC_{inf}); absorption rate constant (k_{abs}); elimination rate constant (k_{el}); absorption half-life ($t_{1/2abs}$); elimination half-life ($t_{1/2el}$), expressed as the harmonic mean; volume of distribution, not corrected for absolute oral bioavailability (V_d/F_{abs}); total body clearance, scaled by absolute oral bioavailability (Cl/F_{abs}); maximum plasma concentration (C_{max}) and time to C_{max} (t_{max}). For lung tissue, AUC_{inf}, k_{el}, $t_{1/2el}$, C_{max} and t_{max} were calculated in a similar way.

The PK data are expressed as mean ± SD for plasma. For lung and PELF a sparse sampling protocol was applied and values are expressed as mean.

The PK/PD index calculated was the time the plasma concentrations remained above the MIC (T>MIC), defined as the cumulative percentage of time over a 24-hour period that the drug concentrations exceed the MIC and should be more than 40% (Hesje et al., 2007).

The areas under the curve (AUC) of the FF concentration-time curve studied under the different housing and feeding conditions were analysed by the Kruskal Wallis test. A p-value below 0.05 was considered statistically significant. The AUC was calculated by the linear trapezoidal rule. These statistical analyses were performed using SPSS Statistics 22 (IBM SPSS software, New York, USA).

Results

Stability of FF in drinking water

The mean (± SD) FF concentration in the medicated drinking water just after preparation was 100.2% (± 0.50%) of the theoretical concentration, indicating a good homogeneity. After 24 h, the FF concentration was 100.4% (± 0.96%) of the initial concentration (t = 0 h), confirming the excellent stability of FF in the drinking water.

MIC evaluation

The *in vitro* activity of FF against 38 *O. rhinotracheale* isolates was tested. Following MIC values were obtained: 0.5, 1.0, 2.0 and 4.0 µg/mL in respectively 3 (7.9%), 32 (84.2%), 2 (5.3%) and 1 (2.6%) of the 38 isolates tested. The MIC_{50} and MIC_{90} values were both 1 µg/mL (Fig. 1).

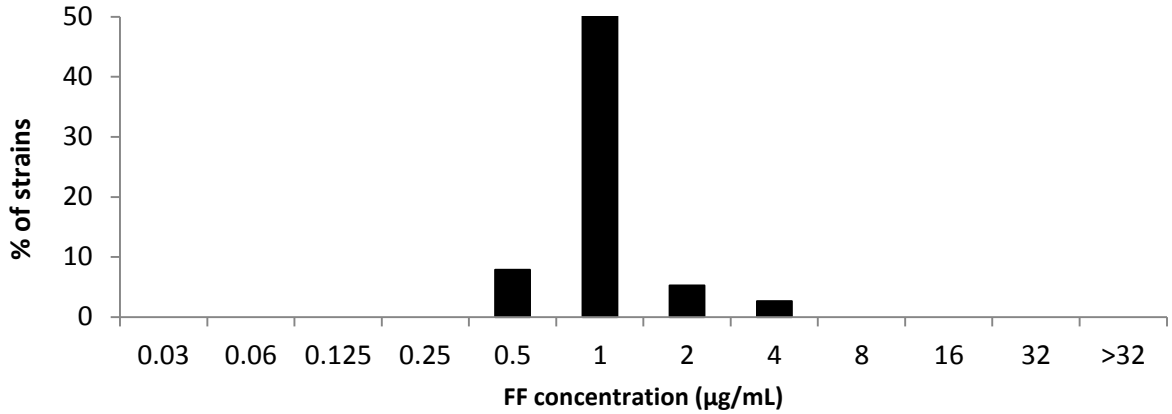

Figure 1. Minimum inhibitory concentration (MIC) distribution of FF in 38 isolates of *Ornithobacterium rhinotracheale*. The MIC_{50} and MIC_{90} were both set at 1 µg/mL.

PK/PD – single oral bolus vs. continuous drinking water administration

In the experiment with continuous drinking water, a reduction of the water consumption during the treatment period was observed (5.35 ± 0.21 L/kg), in comparison with the acclimatization period (8.12 ± 0.37 L/kg).

The plasma concentration-time profiles of both the oral bolus and continuous experiment are depicted in Figure 2. The concentrations during continuous administration were nearly constant during 5 days, followed by a fast elimination when drinking water medication stopped. The C_{max} was much higher after bolus administration and was reached after one hour. During the elimination phase, at the time point of 8 h, a slight rise in plasma concentration can be observed. After 24 h, all plasma concentrations were below the LOQ.

Table 2 presents the PK characteristics of FF in plasma, lung and PELF. FF was rapidly absorbed in plasma and distributed to the respiratory tract (mean k_{abs} = 4.64 h^{-1} in plasma; mean t_{max} is 1 h in plasma, lung tissue as well as in PELF). The mean $t_{1/2el}$ in plasma and lung tissue was similar, 6.27 h and 5.96 h respectively, whereas it was slightly higher in PELF, 8.70 h. In plasma, the mean C_{max} is twice as high as in the lung tissue, 4.26 µg/mL and 2.64 µg/g respectively. The mean concentration in PELF was much lower, i.e. at 0.39 µg/mL.
After a single oral bolus, the FF concentration in plasma and lung tissue exceeded the MIC_{90} for 16.2 and 12 h, respectively.

The mean concentrations in plasma, lung tissue and PELF are shown in Figure 3. Figure 4 shows the individual curves. During drinking water medication, the concentrations in plasma and lung tissue increased, with lung/plasma ratios above 1 (Table 3). After treatment, from day 6 onwards, no concentrations could be detected in plasma and only very low concentrations in lung tissue. The concentrations in PELF were at all time points below the LOQ. After oral bolus administration, the concentrations in plasma, lung tissue as well as PELF were higher compared to drinking water medication. Although, only at 6 and 24 h the lung concentration were higher than the plasma concentrations.

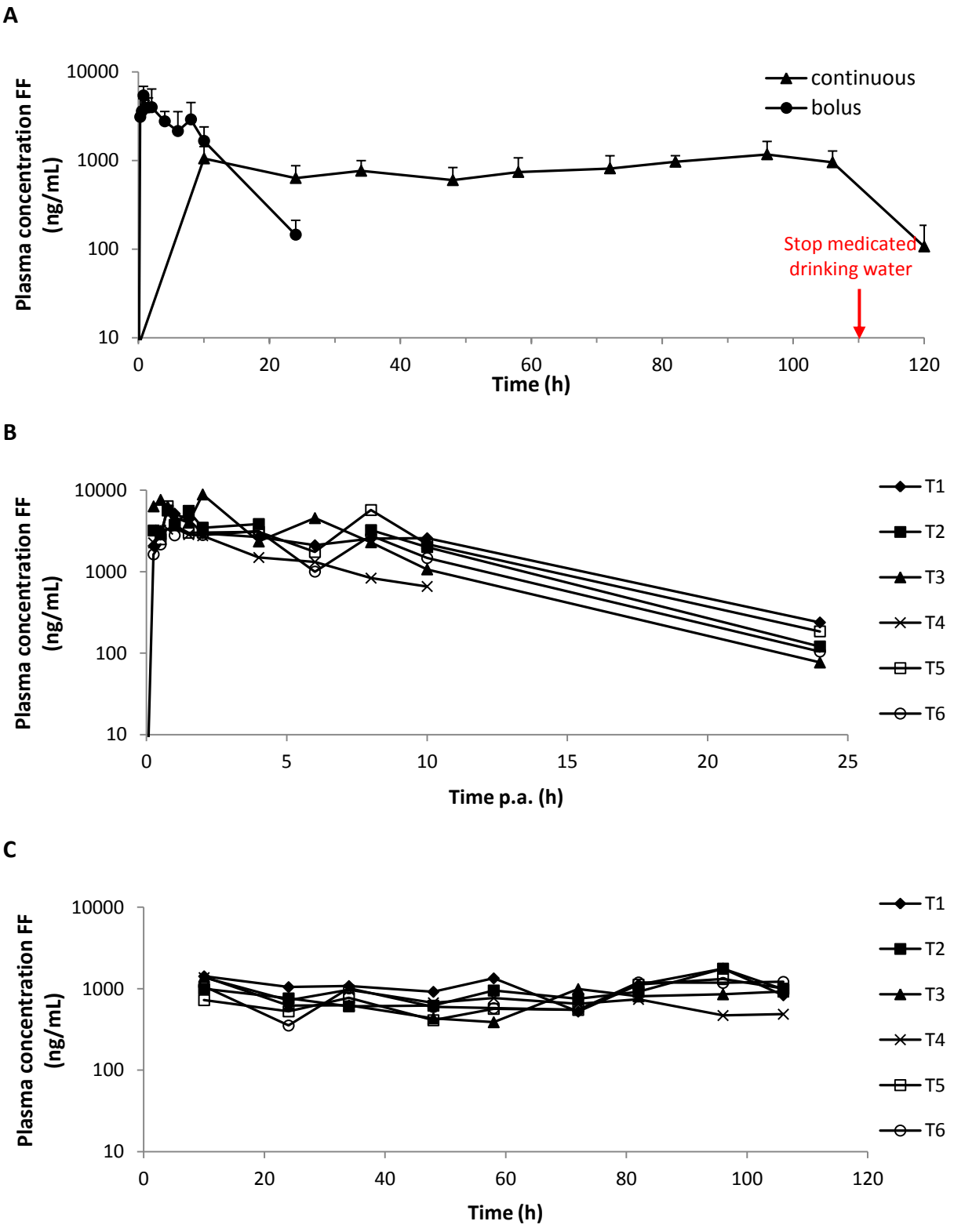

Figure 2. Panel A: Mean (+ SD) plasma concentration (log scale) versus time curve of florfenicol (FF) in turkeys, after either 5-day continuous oral administration of FF via medicated water at a target dose of 30 mg/kg BW/day (n=6, continuous experiment, ▲) or a single oral bolus administration of FF at a dose of 30 mg/kg BW (n=6, oral bolus experiment, ●). Panel B: Individual plots of 6 turkeys after bolus administration. Panel C: Individual plots of 6 turkeys during drinking water medication.

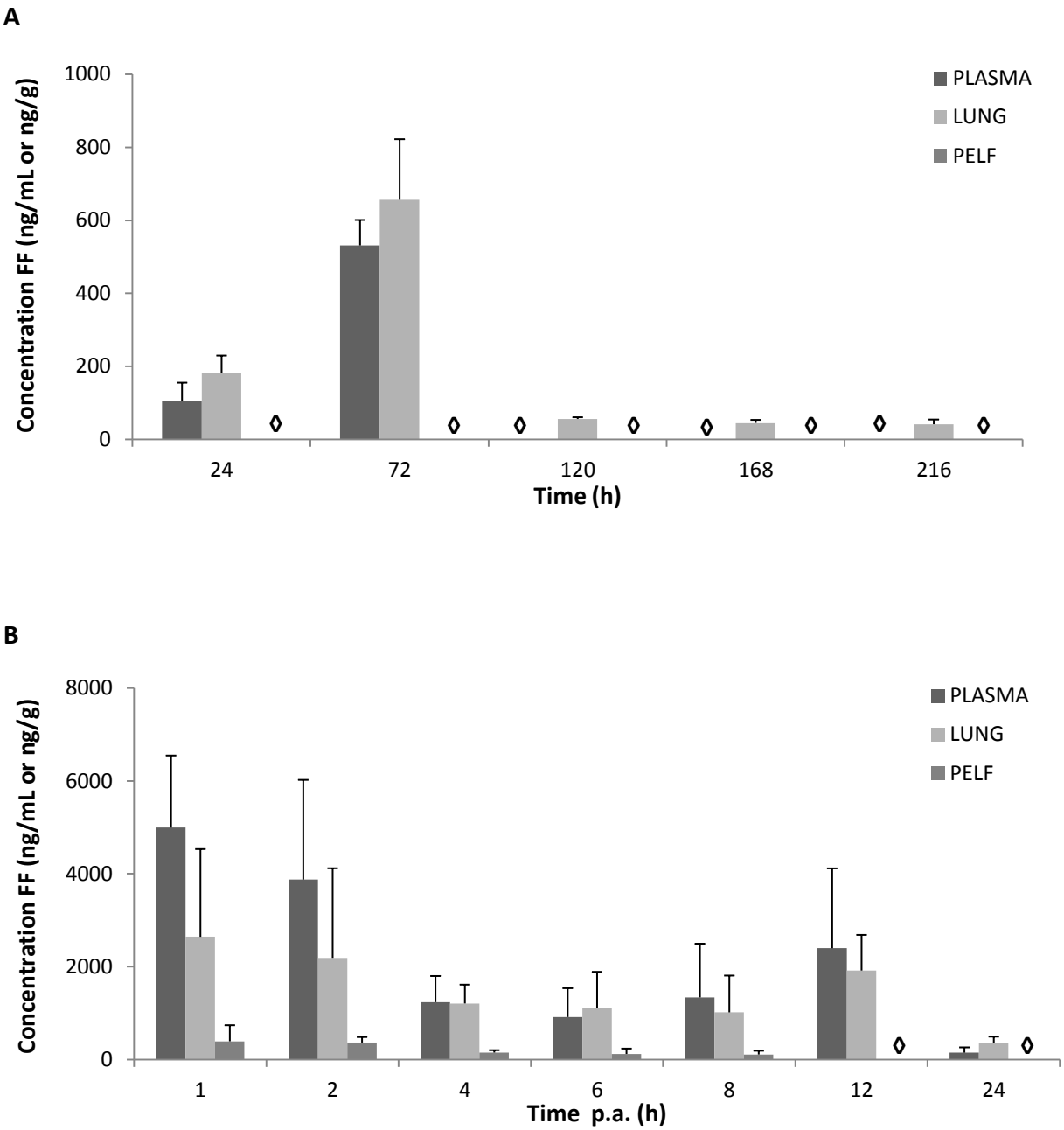

Figure 3. Mean (+SD) plasma, lung tissue and PELF concentrations of florfenicol (FF) in turkeys, after either a 5-day continuous oral administration of FF via medicated water at a target dose of 30 mg/kg BW/day (panel A) or a single oral bolus administration of FF at a dose of 30 mg/kg BW (panel B). At each time point, four (continuous drinking water) or six (oral bolus) turkeys were taken into account. Values below the LOQ are indicated by ◊. At 36 and 48 h after oral bolus, all concentrations were below the LOQ and are not presented.

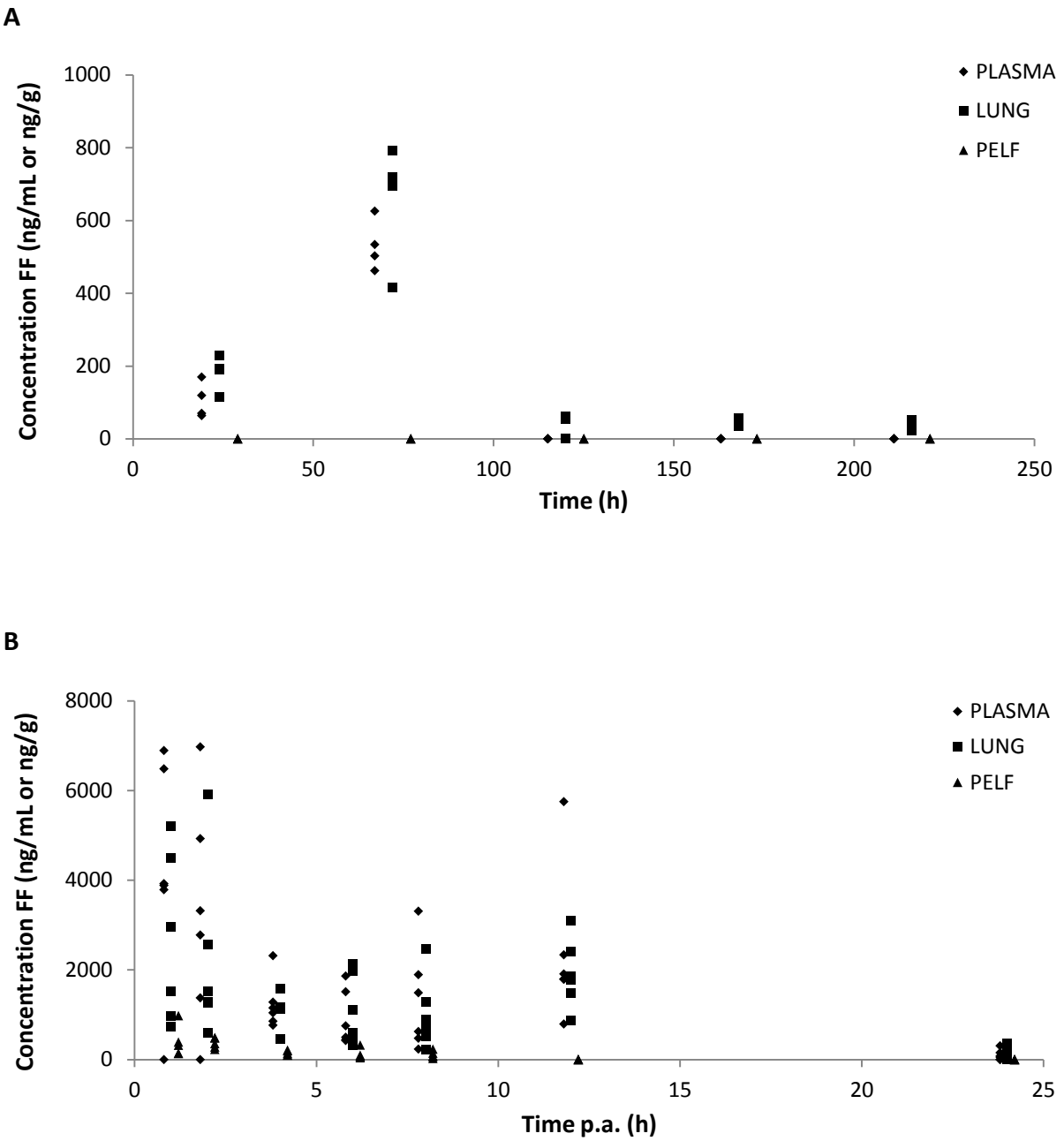

Figure 4. Individual plasma, lung tissue and PELF concentrations of florfenicol (FF) in turkeys, after either a 5-day continuous oral administration of FF via medicated water at a target dose of 30 mg/kg BW/day (panel A) or a single oral bolus administration of FF at a dose of 30 mg/kg BW (panel B). At each time point, six (oral bolus) or four (continuous drinking water) turkeys were sampled.

Table 2. Pharmacokinetic properties of florfenicol in turkey poults after oral (PO) bolus administration of 30 mg/kg body weight, in plasma (n=6), lung tissue and PELF (both n=6 at each time point). Results are presented as mean ± SD (plasma) or mean (lung and PELF).

Parameter	Units	Plasma	Lung	PELF
AUC_{inf}	h.µg/mL or h.µg/g	48.56 ± 18.76	32.63	2.97
k_{abs}	h^{-1}	4.64 ± 3.24	-	-
k_{el}	h^{-1}	0.011 ± 0.05	0.12	0.08
$t_{1/2\ abs}$	h	0.15 [A]	-	-
$t_{1/2\ el}$	h	6.27 [A]	5.96	8.70
V_d/F_{abs}	L/kg	6.75 ± 1.56	-	-
Cl/F_{abs}	L/kg/h	0.74 ± 0.42	-	-
t_{max}	h	1.02 ± 0.39	1.00	1.00
C_{max}	µg/mL or µg/g	4.26 ± 1.30	2.64	0.39

AUC_{inf}, the area under the plasma concentration-time curve from time 0 to infinity; k_{abs}, absorption rate constant; k_{el}, elimination rate constant; $t_{1/2abs}$, half-life of absorption; $t_{1/2el}$, half-life of elimination; V_d/F_{abs}, volume of distribution (not corrected for the absolute oral bioavailability); Cl/F_{abs}, clearance (scaled by absolute oral bioavailability); t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration.
A: harmonic mean

Table 3. The mean concentration ratios of lung/plasma and pulmonary epithelial lining fluid (PELF)/plasma after oral bolus or drinking water medication during 5 days of florfenicol at a dose of 30 mg/kg BW.

Oral bolus			Continuous drinking water		
Time	**Lung/Plasma**	**PELF/Plasma**	**Time**	**Lung/Plasma**	**PELF/Plasma**
1 h	0.64 ± 0.47	0.12 ± 0.10	24 h (day 2)	2.01 ± 1.01	ND
2 h	0.27 ± 0.21	0.24 ± 0.33	72 h (day 4)	1.27 ± 0.42	ND
4 h	0.79 ± 0.53	0.55 ± 1.03	120 h (day 6)	ND	ND
6 h	1.16 ± 0.24	0.15 ± 0.05	168 h (day 8)	ND	ND
8 h	0.91 ± 0.35	0.12 ± 0.04	216 h (day 10)	ND	ND
12 h	0.98 ± 0.45	0.08 ± 0.03			
24 h	1.09 ± 0.31	ND			

ND, not determined

PK/PD – different housing and feeding conditions

The inclusion rate of FF in the medicated drinking water was determined on the basis of mean BW and water uptake per group, and it was between 67.5 and 144.3 mg/L. The daily water intake remained mainly constant before, during and after the treatment in the three groups (mean ± SD: 1.69 ± 0.13 L), with the exception of group 16/8R that drank less on day 5. The mean effective drug intake ranged from 28.2 to 33.1 mg/kg BW/day for all groups. However, on day 5 group 16/8R received only 24.4 mg/kg BW.

The mean plasma concentration-time profiles of FF and the MIC-value are depicted in Figure 5A and 5B for day 1 and day 5, respectively. Remarkable is that on day 1, almost all animals of the 20/4L group had a drop in FF plasma concentration (<400 ng/mL) between 5 and 10 h after the start of medication (5/6 turkeys), while none of the other groups displayed these low FF concentrations. However, this decline resulted not in a significant difference of the AUC between the groups. On day 5, a significant difference was observed between 16/8L and 16/8R. This was probably due to the reduced water intake.

On day 1, plasma concentrations above the MIC could be seen for 37.7%, 63.5% and 53.1% of a 24 h interval for respectively 20/4L, 16/8L and 16/8R groups. However, on day 5 group 20/4L never reached the MIC and groups 16/8L and 16/8R exceeded the MIC for 47.9% and 21.5% of a 24 h interval, respectively.

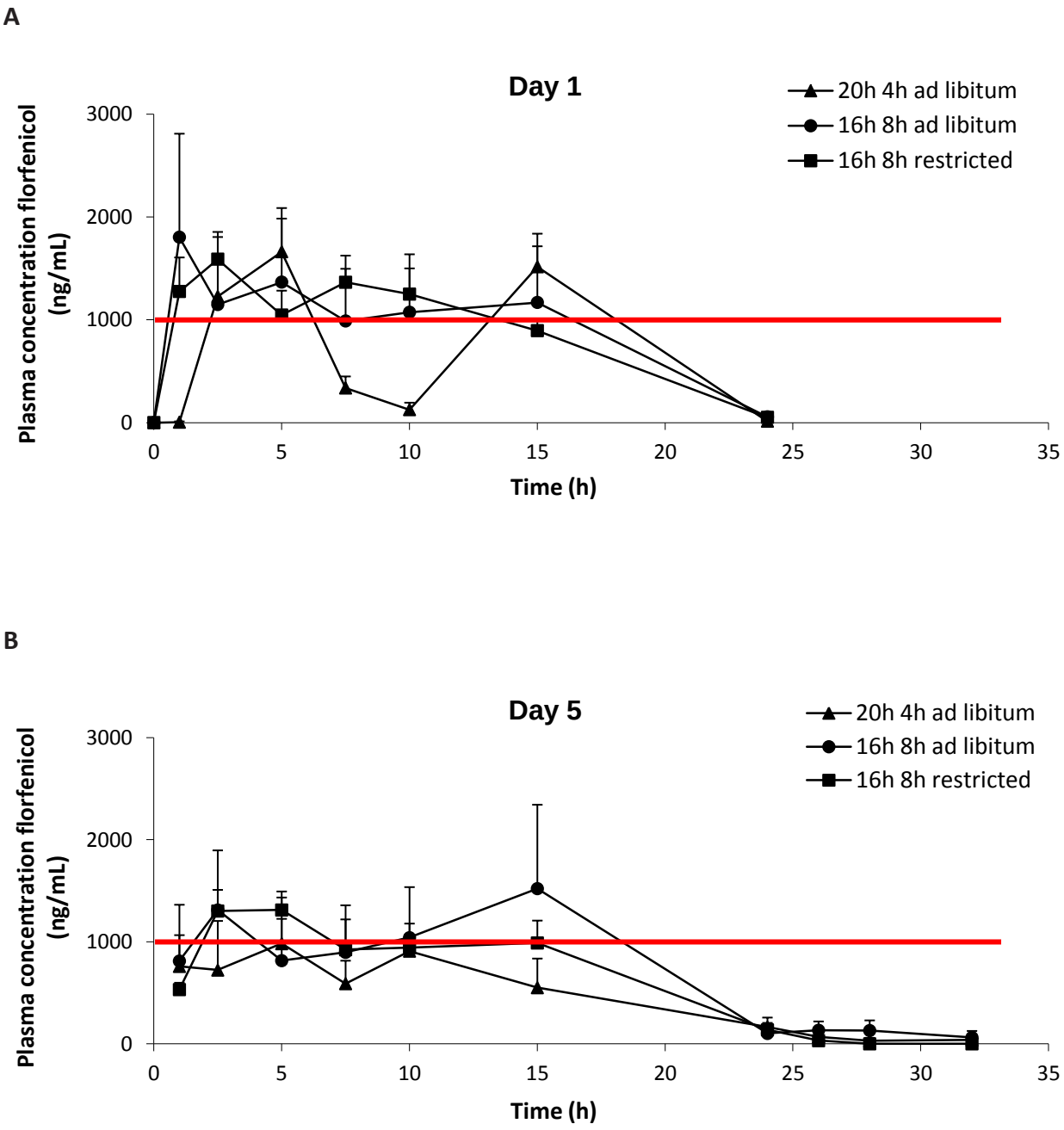

Figure 5. Mean (+ SD) plasma concentration–time profiles of florfenicol in group 20/4L (20h light and 4 h dark, fed *ad libitum*, ▲), group 16/8L (16 h light and 8 h dark, fed *ad libitum*, ●), group 16/8R (16 h light and 8 h dark, fed *ad libitum* from 1h after lighting, ■) on day 1 (A) and day 5 (B) of a 5-day continuous oral administration of florfenicol via medicated water (target dose: 30 mg/kg BW/day). The red line indicates the MIC_{90} value of 1 µg/mL.

Discussion

Notwithstanding FF may be used to treat turkeys from respiratory infections and PK studies in the species of interest are essential, no data on PK characteristics of FF in respiratory tissue of turkeys have been published before. A single oral bolus experiment was performed to determine the concentrations in plasma, lung and PELF and to calculate the corresponding PK characteristics. On the other hand, also a continuous drinking water medication experiment was carried out to represent the treatment in field conditions, determining the concentrations and PK characteristics in these body fluids and tissue. An important drawback of FF, as active pharmaceutical ingredient to use in medicated drinking water, is the low aqueous solubility. Only after stirring and sonication, the drug was dissolved in the drinking water. As already reported, drug intake can be affected by many factors using this way of oral medication. In this study, the influence of two important parameters, namely photoperiod and prandial status on FF uptake using drinking water administration, has been tested. The results demonstrated an important influence of the photoperiod on the PK of FF. On the other hand, there was no effect of the feeding schemes on the disposition of the antibiotic.

MIC evaluation

This is the first study to evaluate the *in vitro* susceptibility of several *O. rhinotracheale* strains to FF. Also no susceptibility breakpoints have been defined yet. The very strict range of *O. rhinotracheale* MIC values reported for the 38 isolates suggested that 1 µg/mL might be the MIC value of the wild type of this bacterium. The unimodal distribution of the MIC values suggests there is no indication for acquired antimicrobial resistance. As the evaluated bacterial population is maybe not large enough, further studies are necessary to confirm these data.

Plasma pharmacokinetics

After a single oral bolus of 30 mg/kg BW, FF showed a fast absorption. This is in accordance with other studies in avian species after an oral FF bolus of the same dose, mean plasma t_{max} varied from 0.30 to 2.00 h (Afifi and Abo El-Sooud, 1997; Shen et al., 2003; Switala et al., 2007; Chang et al., 2010; Abu-Basha et al., 2012). Also in pigs, the t_{max} was similar, namely

1.50 h, after a single oral bolus (Jiang et al., 2006). The mean C_{max} in plasma determined in this study was lower compared to Switala et al. (2007), 4.26 and 12.25 µg/mL, respectively. The same was observed for the mean AUC, 48.56 and 77.62 µg.h/mL respectively. The results of both parameters suggest a lower oral bioavailability of the used active substance. In Leghorn and Taiwan native chickens (Chang et al., 2010), the plasma concentrations were similar to turkeys as reported by Switala et al. (2007), while our results were comparable with those in broiler chickens (Afifi and Abo El-Sooud, 1997; Shen et al., 2003).

With a V_d above 1 L/kg BW, FF is moderate distributed extravascular. The V_d of FF is at variance between different bird species, with a V_d ranging from 1.06 L/kg BW in turkeys, over around 5 L/kg BW in quails, pigeons, ducks and broiler chickens, up to 8.70 L/kg BW in Japanese quails (Afifi and Abo El-Sooud, 1997; El-Banna, 1998; Switala et al., 2007; Ismail and El-Kattan, 2009; Koc et al., 2009b). Although plasma protein binding was not determined in this study, many others reported a low binding for FF in different animal species, < 25% (Adams et al., 1987; Afifi and Abo El-Sooud, 1997; Abd El-Aty et al., 2004). This low extent is consistent with the high V_d.

The elimination process is expressed by the total body Cl and consequently also partly by $t_{1/2el}$. Since no IV bolus was administered, Cl was not corrected for the absolute bioavailability (F_{abs}). Therefore, the mean Cl found in our study (0.74 L/kg/h) could be lower. There is a wide range in Cl value among avian species, depending on their BW, from 0.3-0.6 L/kg/h in larger birds (turkey and Muscovy ducks) towards 1.6 L/kg/h in broiler chickens, 3.9 L/kg/h in pigeons and 5.3 L/kg/h in quails (Afifi and Abo El-Sooud, 1997; El-Banna, 1998; Switala et al., 2007; Ismail and El-Kattan, 2009). A mean plasma $t_{1/2el}$ of 6.27 h in turkeys was comparable with that of Muscovy ducks (El-Banna, 1998), whereas it was twice as high as reported in chickens (Afifi and Abo El-Sooud, 1997; Shen et al., 2003; Ismail and El-Kattan, 2009). Also Switala et al. (2007) found a lower $t_{1/2el}$ value, 3.76 h in turkeys. After 24 h post administration, almost all FF was eliminated from the body.

Regarding the plasma concentration-time profile of the continuous drinking water medication, the plasma concentrations balanced around 1 µg/mL. After stopping the medicated drinking water, FF was rapidly eliminated from the plasma and target tissues.

Despite the significant difference between 16/8L and 16/8R on day 5, we considered that the two different feeding schemes have no influence on the plasma concentration as no differences were observed at day 1. The differences on day 5 could be assigned to the lower

water intake of group 16/8R. Therefore, no differences in plasma concentrations between 16/8L and 16/8R have been pointed out in this study, although several studies confirmed the influence of feeding status on the oral bioavailability (Varma et al., 1986; Baert and De Backer, 2006; Shen et al., 2003; Chang et al., 2010). There could be several reasons for these findings. First of all, the experiments were performed in different species (chicken vs. turkey). Also the way of administration is very important. In contrast to previous studies whereby the chickens received a bolus of FF, we administered the drug via continuous drinking water medication. At last, the restricted and the *ad libitum* group received both 16 h of light, which was demonstrated as a good photoperiod. Consequently, all turkeys showed a rest period before the light was switched on (24 h), suggesting that they did not eat during the dark period.

As can be seen in Figures 2 and 4, it is expected that the groups which received FF *via* the drinking water, have high interanimal variability. The variable intake of medicated water can be the result of the pecking order, stage of sickness or accessibility to water. Only a few studies reported information about drinking behaviour in poultry (Ross and Hurnik, 1983; Puma et al., 2001). However, also after a single oral bolus, high variability between the individual turkeys occur. Consequently, the observed interindividual differences are probably the result of a variation in absorption, distribution, and elimination of the drug.

Lung and PELF pharmacokinetics

Despite that the concentrations of FF in tissues have been reported in avian (Anadón et al., 2008; Chang et al., 2010) as well as mammalian species (Lane et al., 2008), only one study described the PK characteristics of FF in lung tissues. After intramuscular administration of 20 mg FF/kg BW to pigs (Liu et al., 2003), the mean C_{max} and t_{max} of FF in turkey lung tissue was comparable with pigs, 2.64 µg/g – 1 h and 2.46 µg/g – 2 h for turkey and pig, respectively. This confirms the rapid distribution from plasma to lung tissue. However, a great discrepancy can be observed between turkey and pig concerning the elimination from lung tissue. In pigs the $t_{1/2el}$ was 38.5 h whereas in turkeys, the mean $t_{1/2el}$ for lung tissue was 5.96 h. A possible explanation could be the infection status of the animals. In the study of Liu et al. (2003), the pigs were experimentally infected with *Actinobacillus pleuropneumoniae* and this could enhance the affinity of the lung tissue for FF.

To compare the concentrations in either lung tissue and PELF to the plasma concentrations, lung/plasma and PELF/plasma ratios were calculated. For the continuous drinking water experiment, the lung/plasma concentration was above 1 during the treatment, affirming the high affinity for the respiratory tract. Throughout the medication period, the FF lung concentrations increased. After stopping the treatment, only low FF lung concentrations and no plasma concentrations could be detected. In all PELF samples, the FF concentration was below the LOQ.

After the oral bolus administration, the lung/plasma ratio was above 1 only at 6 h and 24 h. This would imply a more rapid elimination from plasma compared to lung tissue, although the $t_{1/2el}$ of plasma and lung are comparable. After 8 h, the plasma concentrations increased and this also reflected a higher lung concentration at 12 h. This phenomenon could be explained by enterohepatic circulation of FF (Pasmans et al., 2008). The high concentration of FF in bile from chickens confirms this suggestion (Afifi and Abo El-Sooud, 1997). After multiple oral doses of 30 mg/kg BW/day for 5 successive days, Afifi and Abo El-Sooud (1997) found detectable lung concentrations (20 µg/g) until 48 h after the last dose. The C_{max} reported in that study was similar to the mean C_{max} in the present study, 2.80 and 2.64 µg/g respectively. In contrast to continuous drinking water medication, FF was detectable in PELF after single bolus administration. However, the PELF/plasma ratios were very low.

This low FF concentrations in PELF could be due to the collection method of PELF and/or to the anatomy and physiology of the respiratory tract of birds (Watteyn et al., 2015).

Plasma and tissue PK/PD – oral bolus vs. continuous drinking water administration

After a single oral bolus, the time above the MIC_{90} was 67.4% and 50% of a 24 h interval in plasma and lung tissue, respectively, but PELF concentrations never exceeded the MIC_{90}. On the contrary, during drinking water treatment the lung and PELF concentrations were never above the MIC_{90}. Also the plasma concentrations were just below the MIC_{90}. This could be due to a reduction of the water consumption during the treatment period compared to the acclimatization period. The reason for this reduction is unknown. Consequently, the postulated dosage of FF of 30 mg/kg BW/day was not reached, only 26.3 ± 3.12 mg/kg BW/day.

Plasma PK/PD – different housing and feeding conditions

During the continuous drinking water medication, the plasma concentrations were around the MIC.

In the continuous drinking water medication experiment with respect to the photoperiod, the two groups with 16 h of light fulfilled to the criterion of a T>MIC of minimum 40% on day 1 (63.1% and 53.1% for 16/8L and 16/8R, respectively). No significant difference between the two feeding schemes were observed. The group 20/4L displayed a very irregular plasma concentration versus time curve on day 1, with a limited time period above MIC (37.7%) and a drop in plasma concentration at 8 and 10 h after the start of the administration of the medicated drinking water. On day 5, the group 16/8R did not achieve 40% (only 21.5%), but this can be related due to a reduced water uptake with unknown reason. From these plasma concentration-time curves, it is obvious that the photoperiod 20/4L is unfavourable to reach sufficient plasma levels. Furthermore, it has to be mentioned that this light scheme is no longer allowed in poultry-rearing, a minimum of 6 h of darkness is required (Anonymous, 2007). Classen et al. (1994) demonstrated that turkeys given a gradually increased light pattern ate and drank more frequently compared to turkeys provided constant light. Also other studies in chickens and turkeys have already related extreme photoperiods with a change in feeding cycles (Newberry, 1992; Brown et al., 2008; Lewis et al., 2009). Diurnal species, like turkeys, eat less in dark than in light conditions, resulting in longer between-feeding intervals (Howie et al., 2010). As feeding uptake in birds is strongly connected with water uptake, changes in photoperiod can easily affect the drinking water uptake. Santos et al. (1997) have reported the same effect during the administration of doxycycline, and underlined the importance of photoperiod during continuous water administration of drugs, especially if the applied drug has a short half-life of elimination. The unusual plasma concentration-time curve in the 20/4L group could be related to an atypical water uptake, as a result of a too short dark period.

Conclusion

FF was very rapidly absorbed and distributed to the lung tissue after a single oral bolus in turkeys. The presented PK and PD characteristics motivate the use of FF as a treatment for respiratory diseases in turkeys, such as *O. rhinotracheale* infections. Ideally, the *in vivo* efficacy of FF against *O. rhinotracheale* should be determined in an experimental infection model.

This study confirms also the negative influence of a light scheme with 20 h of light in drug administration as reported by Santos et al. (1997). The continuous administration of 30 mg of FF/kg BW via the drinking water for 5 days at a photoperiod of 16 h resulted in better PK/PD indices compared to a photoperiod of 20 h. Replacing the drinking water once daily would be sufficient as FF is stable over a 24 h period.

However, FF as such is not applicable for drinking water medication as the water solubility is very low (only 1 mg/mL), which implies an elaborate preparation of the medicated water. Therefore, an appropriate water soluble formulation would be preferable.

Acknowledgements

The authors wish to thank Jelle Lambrecht, Matteo Stecca and Anja Van den Bussche for their excellent laboratory assistance. For the aid in the animal experiment, we thank Gunther Antonissen, Nathan Broekaert, Thomas De Mil, Sophie Fraeyman, Elke Gasthuys, Joline Goossens, Elke Plessers, Charlotte Watteyn and Heidi Wyns.

Chapter 1.2

Pharmacokinetics and pharmacodynamics of gamithromycin in plasma, lung tissue and pulmonary epithelial lining fluid in turkeys, after a single bolus

Adapted from:

Watteyn A., Devreese M., De Baere S., Wyns H., Plessers E., Boyen F., Haesebrouck F., De Backer P. and Croubels S. (2015). Pharmacokinetic and pharmacodynamic properties of gamithromycin in turkey poults with respect to *Ornithobacterium rhinotracheale*. *Poultry Science*, 94, 2066-2074.

Abstract

The macrolide gamithromycin (GAM) has the ability to accumulate in tissues of the respiratory tract. Consequently, GAM might be a suitable antibiotic to treat bacterial respiratory infections in poultry, such as *Ornithobacterium rhinotracheale*. As *O. rhinotracheale* infections are common in turkey flocks, the aim of this study was to determine the pharmacokinetic (PK) parameters of GAM in plasma, lung tissue and pulmonary epithelial lining fluid (PELF) of turkeys and to correlate them with pharmacodynamic (PD) characteristics (PK/PD). The animal experiment was performed with 64 turkeys, which received either a subcutaneous (SC, n=32) or an oral (PO, n=32) bolus of 6 mg GAM/kg body weight (BW). GAM concentrations in plasma, lung tissue and PELF were measured at different time-points post administration (p.a.), and PK characteristics were determined using non-compartmental modelling. The mean maximum plasma concentration after PO administration was a ten-fold lower than after SC injection (0.087 and 0.89 µg/mL, respectively), whereas there were no differences in lung concentrations between both routes of administration. However, lung concentrations at day 1 p.a. were significantly higher than plasma levels for both routes of administration (2.22 and 3.66 µg/g for PO and SC, respectively). Consequently, lung/plasma ratios were high, up to 50 and 80 after PO and SC administration, respectively. GAM could not be detected in PELF, although this might be attributed to the collection method of PELF in birds. The GAM minimum inhibitory concentration (MIC) was determined for 38 *O. rhinotracheale* strains with MIC_{50} and MIC_{90} of 2 and >32 µg/mL, respectively. PK/PD correlation for lung tissue demonstrated that the time above the MIC_{90} of the susceptible population (2 µg/mL) was 1 day after PO bolus and 3.5 days after SC administration. The area under the curve (AUC_{last})/MIC ratio for lung tissue was 233 and 90 after SC and PO administration, respectively. To conclude, GAM is highly distributed to lung tissue in turkey poults, suggesting that it has the potential to be used to treat respiratory infections such as *O. rhinotracheale*.

Introduction

Gamithromycin (GAM) is a new generation macrolide antibiotic, belonging to the azalide subgroup. Macrolides are widely used antibiotics in veterinary medicine. A unique feature of these compounds is their ability to accumulate in the respiratory tract (Giguère, 2013). GAM is indicated for the treatment of bovine respiratory disease (BRD) (Baggott et al., 2011), but is currently not registered for use in other species. Nevertheless, the manufacturer has intentions to register the product for treatment of swine respiratory disease (SRD) since just recently, maximum residue levels (MRLs) have been established for porcine species too (EMA, 2015).

In poultry, bacterial infections of the respiratory tract frequently result in economic losses due to an increased mortality and feed conversion rate, a reduced growth and high medical costs (Van Empel and Hafez, 1999). *Ornithobacterium rhinotracheale* is a Gram-negative bacterium causing respiratory symptoms in several bird species. Infections with *O. rhinotracheale* have been treated with several classes of antimicrobials, including β-lactam antibiotics, tetracyclines, fluoroquinolones, florfenicol and macrolides, but with variable outcomes (Marien et al., 2006, 2007; Garmyn et al., 2009a,b, Warner et al., 2009; Agunos et al., 2013; Watteyn et al., 2013b). Several studies demonstrated that the sensitivity of *O. rhinotracheale* to antimicrobials is strain-dependent (Devriese et al., 1995, 2001).

The pharmacokinetic (PK) behaviour of GAM has been studied in cattle (Huang et al., 2010; Giguère et al., 2011), foals (Berghaus et al., 2011), broiler chickens (Watteyn et al., 2013a) and swine (Wyns et al., 2014). However, no data are available for turkey poults, neither for plasma nor for tissues.

GAM has a high volume of distribution (V_d > 20 L) in all investigated species, due to its accumulation in tissues and high affinity for the respiratory tract. Huang et al. (2010) analyzed whole lung homogenate of cattle and reported concentrations that were 250 to 400 times higher than the corresponding plasma concentrations. Also in pulmonary epithelial lining fluid (PELF), the concentrations of GAM were much higher compared to plasma, with a maximum plasma concentration (C_{max}) of 0.43 and 0.33 µg/mL in plasma and 4.16 µg/mL and 2.15 µg/mL in PELF for cattle and foals, respectively (Giguère et al., 2011; Berghaus et al., 2011). This emphasizes the need to quantify the antibiotic in the target pulmonary tissues as well, and not only in plasma.

Since GAM has a spectrum against *O. rhinotracheale*, and combined with the ability to accumulate in pulmonary tissues, it might be used to treat *O. rhinotracheale* infections. Therefore, the aim of the present study was to determine the PK behaviour of GAM in plasma as well as in lung tissue and PELF of turkey poults, and to relate these results to the minimum inhibitory concentration (MIC) values of recent *O. rhinotracheale* isolates.

Materials and Methods

Experimental protocol

Sixty-four 3-week-old female turkey poults with a mean body weight (BW) (± SD) of 0.556 (± 0.057) kg (Hybrid Converter, local commercial turkey farm) were housed according to the requirements of the European Union (Anonymous, 2010). The animals were acclimatized for 4 days and received water and feed *ad libitum*. Feed was withdrawn from 12 h before until 6 h after GAM administration. The turkeys were randomly divided in two groups. Thirty-two animals received a subcutaneous (SC) bolus injection of 6 mg/kg BW GAM in the neck region. The other 32 birds were administered the same dose, but orally (PO) by gavage in the crop. Blood (1 mL) was collected from 5 animals per group by venipuncture of the leg vein into heparinized tubes (Vacutest Kima, Novolab, Geraardsbergen, Belgium) at different time points before (time 0 h) and post administration (p.a.; 0.08, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10 and 12 h, and furthermore once daily in the morning from day 2 (24 h) until day 10 p.a. and once on days 12 and 14). Blood samples were centrifuged at 1500 *x g* at 4 °C for 10 min. Plasma was collected and stored at ≤ -15 °C until analysis.

From each group, four animals were sacrificed at different time points (day 1, 5, 10, 15, 20, 30, 40 and 50 p.a.) to collect plasma, lung tissue and PELF. For that, the birds were anesthetized using a combination of xylazine (Xyl-M 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100, Virbac, Wavre, Belgium), followed by exsanguination. The whole right lung was removed for GAM analysis. The complete left lung was used to collect PELF as described by Bottje et al. (1999). In brief, after weighing the lung, it was lavaged with heparin-saline (200 units heparin per mL of 0.9% saline) at a volume of 2 mL/g lung through a cannula in the first bronchus. The PELF/saline solution was collected in a petri dish and the amount of fluid was measured to determine the recovery, which ranged from

71.2 to 92.9%. The fluid was centrifuged (5250 *x g* for 3 min) to remove red blood cells. Both the lung tissue and PELF were stored at ≤ -15 °C until analysis.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2013/107).

Veterinary drug, analytical standards, chemicals and solutions

Zactran, containing 150 mg GAM/mL (Merial Ltd, North Brunswick, NJ, USA) was used for the animal experiment. Just before drug administration, it was diluted with *aqua ad injectabilia* up to a concentration of 15 mg GAM/mL.

The analytical standard of GAM and the internal standard (IS), deuterated-GAM (d5-GAM), were kindly donated by Merial Ltd and stored at 2 – 8 °C. Stock solutions of 1 mg/mL of GAM and d5-GAM were prepared in methanol (MeOH) and stored at ≤ -15 °C. Working solutions of 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100 µg/mL of GAM were prepared by appropriate dilution in HPLC water. Working solutions of 1.0 and 10.0 µg/mL of the IS were prepared in HPLC water by appropriate dilution of the stock solution. The working solutions of GAM and IS were stored at 2 – 8 °C.

The solvents used for HPLC analysis (water and acetonitrile, ACN) were of LC-MS grade and obtained from Biosolve (Valkenswaard, The Netherlands). All other solvents and reagents were of HPLC grade (water, ACN, MeOH and diethylether) or analytical grade (formic acid, ammonium acetate, sodium hydroxide (NaOH) and ammonium hydroxide) and purchased from VWR (Leuven, Belgium). Millex-GN Nylon (0.20 µm) syringe filters were obtained from Merck Millipore (Overijse, Belgium). Ostro protein precipitation and phospholipid removal 96-well plates (25 mg) were obtained from Waters (Zellik, Belgium). HybridSPE-Phospholipid cartridges (30 mg/mL) were purchased from Sigma-Aldrich (Bornem, Belgium).

Gamithromycin analysis

Sample preparation for the analysis of GAM in turkey plasma, using the Ostro 96-well plates and a validated high performance liquid chromatography method with tandem mass spectrometric detection (LC-MS/MS), was performed as described by Watteyn et al. (2013a) for chicken plasma. Lung and PELF samples were analyzed using a validated LC-MS/MS method by De Baere et al. (2015).

The limit of quantification (LOQ) was 5 ng/mL, 50 ng/g and 20 ng/mL for plasma, lung tissue and PELF, respectively (De Baere et al., 2015).

Minimum Inhibitory Concentration

The MIC of GAM was determined using the agar dilution method, as described in Chapter 1.1. The concentrations of GAM tested ranged between 0.03 and 32 µg/mL.

The determination of the MIC was performed in duplo, with an interval of 3 weeks.

Pharmacokinetic and statistical analysis

Following plasma PK parameters were determined by non-compartmental analysis (WinNonlin 6.3, Pharsight, CA, USA): area under the plasma concentration-time curve from time 0 to the last time point with a quantifiable concentration (AUC_{last}); the AUC from time 0 to infinity (AUC_{inf}); elimination rate constant (k_{el}); elimination half-life ($t_{1/2el}$); volume of distribution (V_d); total body clearance (Cl); C_{max} and time to C_{max} (t_{max}). The relative oral bioavailability (F_{rel}) was calculated according to the following equation: $F_{rel}\ (\%) = \frac{AUC_{last\ PO}}{AUC_{last\ SC}} \times 100$. For lung tissue, AUC_{last}, AUC_{inf}, k_{el}, $t_{1/2el}$, C_{max} and t_{max} were calculated in a similar way. Results below the LOQ were not taken into account.

The plasma PK data are expressed as mean ± SD and were statistically analyzed by the nonparametric Mann-Whitney U test, using SPSS Statistics 22 (IBM, New York, USA). A value of $P < 0.05$ was considered significant. No SD could be calculated for the lung samples, as a sparse sampling protocol was used. Hence, no statistical analysis was performed.

Results

The semi-logarithmic plots of the mean and individual plasma concentration-time curves of GAM after SC and PO administration are depicted in Figure 1, while Figure 2 shows the comparison between the concentration-time curves in plasma and lung tissue.

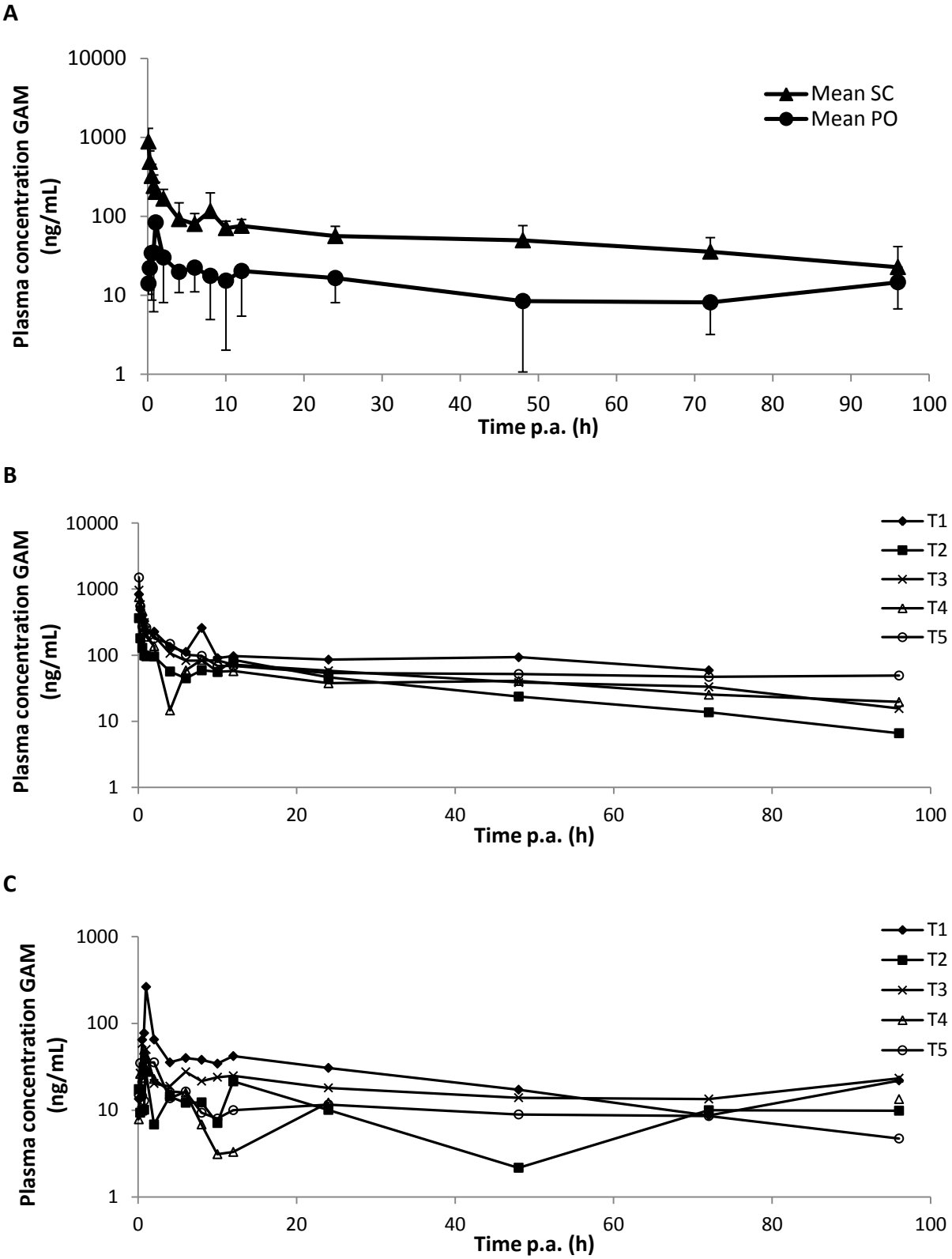

Figure 1. Panel A: Mean (± SD) plasma concentration versus time curve of gamithromycin (GAM) in turkeys, after subcutaneous (SC, n=5) or oral (PO, n=5) administration of 6 mg/kg BW GAM. Panel B: Individual plots of 5 turkeys after SC bolus administration. Panel C: Individual plots of 5 turkeys after PO bolus administration. p.a., post administration.

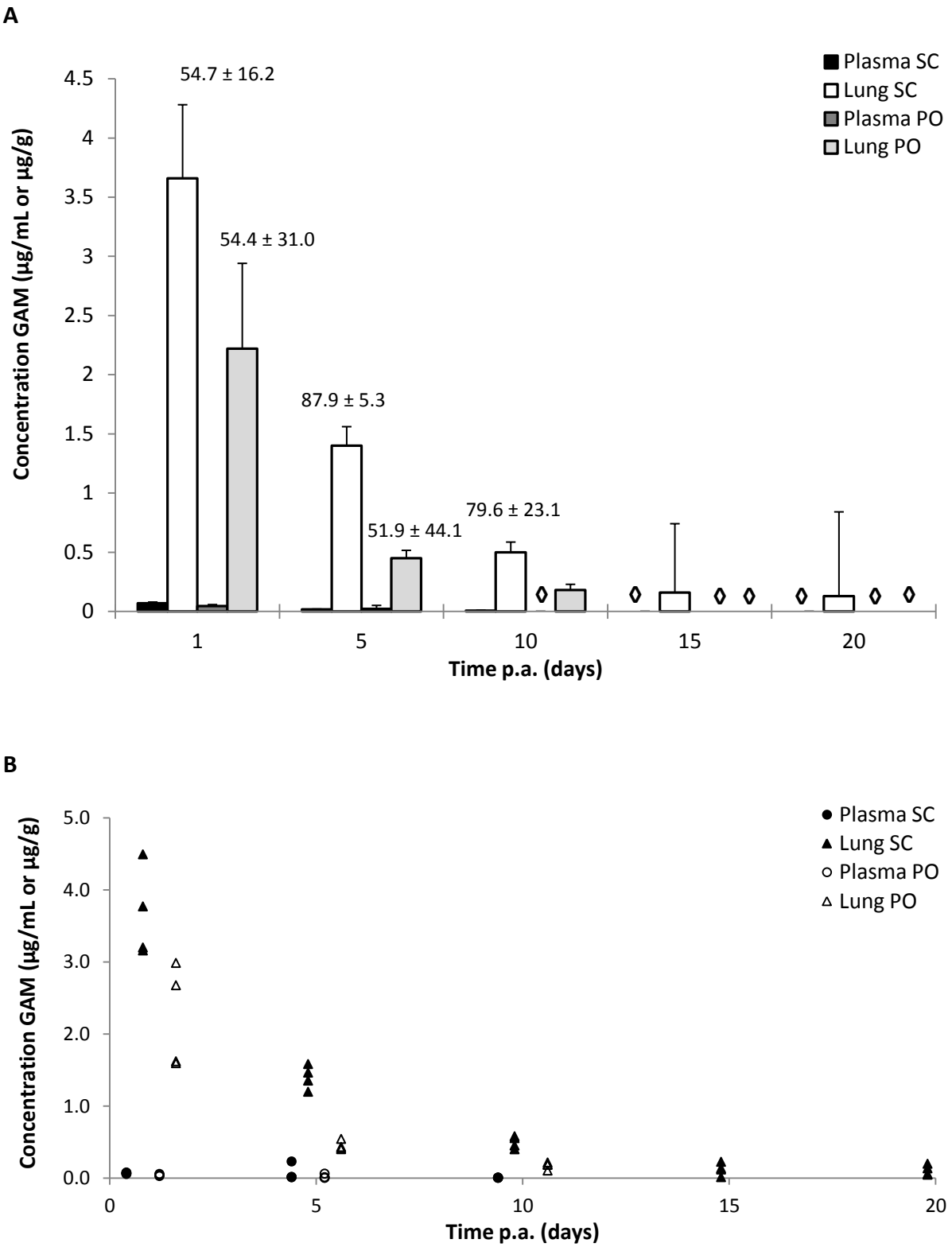

Figure 2. Panel A: Mean (+SD) plasma and lung tissue concentrations of gamithromycin (GAM) in turkeys, after subcutaneaous (SC) or oral (PO) administration of 6 mg/kg BW GAM. At each time point, four turkeys were taken into account. Values below the LOQ are indicated by ◊. The lung/plasma concentration ratios are displayed above the corresponding route of administration. p.a., post administration. Panel B: Individual plasma, lung tissue and PELF concentrations of GAM in turkeys after either a SC or PO single bolus administration.

Table 1 shows the main PK properties of GAM for plasma and lung tissue. As can be observed, the AUC_{last} as well as the AUC_{inf} after PO administration for both plasma and lung tissue were much lower than after SC administration, with significant difference in plasma ($P < 0.01$ and $P < 0.05$ for AUC_{last} and AUC_{inf}, respectively.). After PO administration, C_{max} in plasma was a ten-fold lower than after SC administration (0.087 and 0.89 µg/mL, respectively). Nevertheless, this discrepancy between SC and PO was not seen in the lung tissue (C_{max} of 2.22 and 3.66 µg/g after PO and SC administration, respectively). The V_d and Cl were corrected for the relative oral bioavailability (F_{rel} = 25.0%), and were not significantly different between routes of administration. Consequently, the $t_{1/2\ el}$ in plasma for both routes of administration were not significantly different (Table 1 and Figure 1).

As can be seen in Figure 2, the lung/plasma concentration ratios of GAM were up to 87.9. No plasma concentrations were detected from 10 and 15 days onwards after PO and SC administration, respectively.

The concentration of GAM in all PELF samples was below the LOQ of 20 ng/mL.

The MIC values of the 38 *O. rhinotracheale* isolates ranged from 0.25 to >32 µg/mL, namely 0.25, 0.5, 1.0, 2.0, 4.0 and >32 µg/mL in respectively 1 (2.6%), 4 (10.5%), 9 (23.7%), 7 (18.4%), 3 (7.9%) and 14 (36.8%) of the evaluated strains (Figure 3). For the type strain LMG 9086, the MIC was 0.5 µg/mL. The MIC_{50} and MIC_{90} were 2 and >32 µg/mL, respectively. The control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 showed a MIC of >32 and 4 µg/mL, respectively.

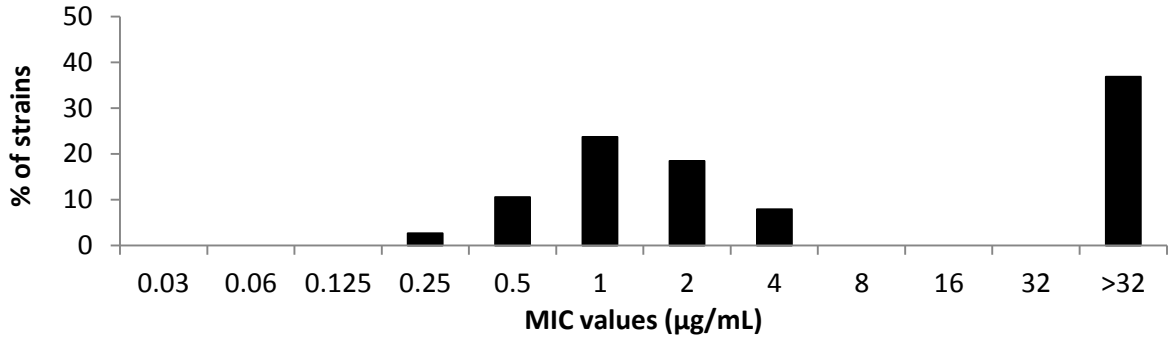

Figure 3. Minimum inhibitory concentration (MIC) distribution of gamithromycin in 38 strains of *Ornithobacterium rhinotracheale*. The MIC_{50} and MIC_{90} were 2 and >32 µg/mL, respectively.

Table 1. The mean pharmacokinetic properties of gamithromycin in turkey poults after subcutaneous (SC) and oral (PO) bolus administration of 6 mg/kg BW, in plasma (n=5) and lung tissue (n=4 at each time point). Results are presented as mean ± SD.

Parameter	Units	Plasma		Lung	
		SC	PO	SC	PO
AUC_{last}	h.µg/mL or h.µg/g	5.14 ± 1.62	1.28 ± 0.72*	452.02	165.63
AUC_{inf}	h.µg/mL or h.µg/g	6.85 ± 2.83	2.17 ± 1.30*	466.85	180.75
k_{el}	h^{-1}	0.020 ± 0.0069	0.023 ± 0.20	0.0075	0.012
$t_{1/2\ el}$	h	34.9 A	29.7 A	92.6	59.8
V_d/F_{abs}	L/kg	53.69 ± 19.66	203.52 ± 126.87	-	-
Cl/F_{abs}	L/kg/h	1.02 ± 0.47	3.80 ± 2.37	-	-
t_{max}	h	0.08 ± 0.00	0.85 ± 0.22*	24.0	24.0
C_{max}	µg/mL or µg/g	0.89 ± 0.41	0.087 ± 0.099*	3.66	2.22
F_{rel}	%	-	25.0	-	-

AUC_{last}, area under the plasma concentration-time curve from time 0 to the last time point with a quantifiable concentration; AUC_{inf}, the AUC from time 0 to infinity; k_{el}, elimination rate constant; $t_{1/2el}$, half-life of elimination; V_d/ F_{abs}, volume of distribution (not corrected for F_{abs}); Cl/F_{abs}, clearance (scaled by absolute oral bioavailability); t_{max}, time to maximum plasma concentration; C_{max}, maximum plasma concentration; F_{abs}, absolute bioavailability; F_{rel}, relative oral bioavailability

A: harmonic mean

*: significant difference ($P < 0.05$) in plasma between routes of administration

For macrolides in general, both the time the plasma concentration exceeds the MIC (T>MIC) and the area under the inhibitory curve (AUC/MIC) are taken into account as PK/PD indices. Considering the clear bimodal MIC distribution (Figure 3), the isolates were divided in a susceptible population (MIC between 0.25 and 4 µg/mL) and a resistant population (MIC >32 µg/mL). In this study, the plasma concentrations never exceeded the MIC_{90} of the susceptible population, which was 2 µg/mL. The $T>MIC_{90}$ in lung tissue was approximately 3.5 days and 1 day after SC and PO administration, respectively. The AUC_{inf}/MIC in plasma was 3.43 and 1.09 after SC and PO administration, respectively. For lung tissue, the AUC_{last}/MIC was 233 and 90 after SC and PO administration, respectively.

Discussion

As macrolides, including GAM, are commonly used in cattle to treat BRD, a possible positive effect of GAM to cure an *O. rhinotracheale* infection in turkeys can be put forward. To identify the disposition of GAM in turkeys, a PK study of GAM in plasma, lung tissue as well as PELF was performed. These results were correlated to the MIC of several *O. rhinotracheale* strains in order to establish a pharmacokinetic/pharmacodynamic (PK/PD) correlation.

The commercial formulation of GAM is only indicated for SC use, but as mass medication through drinking water and feed is the most important route of drug administration in poultry, GAM was also given orally as a single bolus in the crop.

Plasma

To the author's knowledge, no plasma PK studies of macrolides in turkeys have been performed. After SC administration, GAM was absorbed very rapidly, with a t_{max} of 0.08 h, whereas t_{max} after oral administration was delayed (0.85 h). This rapid SC absorption was also seen in broiler chickens (Watteyn et al., 2013a). The $t_{1/2\ el}$ of GAM was not significantly different between SC and PO administration (34.9 h and 29.7 h, respectively), and is similar to foals after intramuscular administration of 6 mg/kg BW GAM (39.1 h; Berghaus et al., 2011). Cattle show a longer $t_{1/2\ el}$, around 50 h after SC administration (Huang at al., 2010; Giguère et al., 2011), while pigs eliminate the drug more rapidly after SC injection ($t_{1/2\ el}$ = 18.8 h; Wyns et al., 2014). In contrast with turkeys, chickens have a shorter $t_{1/2\ el}$ after SC

administration (11.6 and 34.9 h for chicken and turkey, respectively), which can be partially attributed to a higher clearance in comparison with turkeys (1.77 and 1.02 L/kg/h for chicken and turkey, respectively; Watteyn et al., 2013a). Notwithstanding the V_d is similar for GAM in cattle, chickens and pigs (around 20 L/kg), in turkeys it was found to be higher (53.69 L/kg) and might thus also be responsible for the longer $t_{1/2\ el}$ seen in turkeys. An explanation for this discrepancy is possible differences in protein binding across species (Rivière et al., 1997). Cl and V_d are not corrected for the absolute SC bioavailability (F_{abs}), as there are no PK parameters available after intravenous (IV) administration in turkeys. Taking into account that GAM is completely absorbed after SC injection in other species, including cattle, chickens and pigs, it can be suggested that it is also the case for turkeys (Huang et al., 2010; Watteyn et al., 2013a; Wyns et al., 2014). Comparing the AUC of GAM after PO and SC administration, this results in a relative bioavailability (F_{rel}) of 25% after PO. When the Cl and V_d are adjusted for this F_{rel}, these parameters have equal values after PO and SC administration.

The maximum plasma concentration after a SC administration of 6 mg/kg BW GAM in turkeys (0.89 µg/mL) is equivalent to the C_{max} reported for cattle and chickens (0.75 and 0.89 µg/mL respectively; Huang et al., 2010; Watteyn et al., 2013a). This value is higher compared to foals (IM administration) and pigs, namely 0.33 and 0.41 µg/mL after administration of the same dose, respectively (Berghaus et al., 2011; Wyns et al., 2014). After an oral bolus, the C_{max} in plasma is remarkably lower (0.087 µg/mL). A possible hypothesis for this difference could be the presence of the microbiota in the crop which could inactivate macrolides (Dutta and Devriese, 1981; Devriese and Dutta, 1984).

Lung

Although plasma concentrations of macrolides are often below the MIC of the pathogen, these drugs are effective in the treatment of respiratory diseases due to high levels of the active substance in target tissues, represented by their high V_d. Therefore, to evaluate the PK/PD correlation of macrolides, it is of great importance to measure drug concentrations in the target tissues. In the present study, high lung concentrations were detected, with lung/plasma concentration ratios between 54.7 to 87.9 after SC injection. This is in accordance with previous reports (Huang et al., 2010; Giguère et al., 2011) where lung/plasma ratios up to 200 were observed after SC administration of GAM in cattle.

Although lower compared to SC administration, high lung/plasma ratios were also observed after oral administration (51.9 – 54.4). Notwithstanding the C_{max} in plasma after PO was a ten-fold lower than after SC administration, this discrepancy was not observed in the lung (3.66 and 2.22 µg/g after SC and PO administration on day 1 p.a.). As macrolides can be considered as time-dependent antibiotics, the AUC is even more important than C_{max}. If the AUC would be a parameter to compare the amount of drug in plasma and lung tissue, this ratio (AUC_{lung}/AUC_{plasma}) remains constant, after SC as well as PO administration (respectively 53.6 and 51.9 on day 1 p.a.; 55.5 and 45.3 on day 5 p.a.). After SC injection, the $t_{1/2\,el}$ of GAM in lung tissue was similar for cattle and turkeys, namely around 90 h (Huang et al., 2010; Giguère et al., 2011), while it was shorter after oral administration (59.8 h).

PELF

Currently, the pathogenesis of *O. rhinotracheale* and the factors determining colonization of the host tissue are still unclear. *O. rhinotracheale* adheres to avian erythrocytes and tracheal cells, behaving as an extracellular pathogen (De Haro-Cruz et al., 2013). In contrast, Zahra et al. (2013) isolated small-colony variants of *O. rhinotracheale*, which persist intracellularly in murine RAW 264.7 macrophages. This new insight is of great importance for a successful treatment with antimicrobials, although it is not clear if *O. rhinotracheale* is also able to persist in avian macrophages. It is most likely that the distribution of GAM varies among the different compartments of the respiratory tract, such as intracellularly in host defense cells (e.g. macrophages), extracellularly and in bronchial fluid (Huang et al., 2010; Giguère, 2013). As in this study whole lung tissue homogenates were analyzed, the mean concentration in all these compartments was measured. Determination of GAM in PELF might give a more accurate prediction as these concentrations are of importance for extracellular pathogens. Therefore, Giguère and Tessman (2011) concluded that measurement of the concentrations of macrolides in PELF would be a better predictor of their efficacy than either lung or plasma concentrations. As macrolides reach high intracellular concentrations, tissue homogenates could overestimate extracellular concentrations in relation to the PELF.

To date, no PK data of macrolides in PELF from poultry are available. Giguère et al. (2011) detected PELF concentrations of GAM in cattle that were much higher than in plasma, but lower than in lung tissue (ratios between 4.7 and 127 for PELF/plasma and between 16 and

650 for lung/plasma). Also in foals, GAM reached high levels in PELF, with PELF/plasma ratios between 4.7 - 70 (Berghaus et al., 2011). Remarkably, in this study no concentrations of GAM above the LOQ could be detected in PELF of turkeys. A possible explanation could be the typical anatomical arrangement of the respiratory system in avian species. The intrapulmonary primary bronchus ramifies in several secondary bronchi and ends in the abdominal air sac. The ventro- and laterobronchi end also in air sacs via *ostia*, while the dorsobronchi give rise to parabronchi. In contrast to mammals, birds have flow-through lungs with a nearly constant volume, in which the gas exchange takes place in the parabronchi. As air sacs act as bellows, they are the sites of volume expansion and move air through the parabronchi (Brown et al., 1997; Fedde et al., 1998; Powell, 2000). These anatomical differences have an influence on the collection method for PELF. In mammals, PELF is collected by intrabronchial administration of saline in live animals, followed by aspiration of the saline solution and a recovery correction based on an endogenous component, such as ureum. In poultry, on the other hand, the bronchi are connected with the air sacs via *ostia*. As a consequence, it is impossible to apply the same technique as in mammals. The used technique in this study was based on a heparin-saline solution to flush the ex vivo lungs, which distributed in the lung and was immediately flushed out of the lungs through these *ostia* as was reported by Bottje et al. (1999). In contrast, Bernhard et al. (2001) used an in situ method in ducks and chickens. The air sacs were ligated and lungs were flushed with saline, followed by aspiration of the fluid. *In vivo* collection in chickens has also been described. After placing the bird on its back, a tubing was threaded down the exteriorized trachea to the bronchi and air was evacuated from the lung. Warm buffer was administered and the fluid sample was aspirated (Holt et al., 2005). The results obtained might therefore be dependent on the collection method.

Another factor related to the discrepancy seen in GAM concentrations in PELF between mammals and turkeys is the different immunology between the two classes, as GAM also distributes in macrophages. The epithelial surface of the mammalian lung is covered by a thin layer of PELF and resident immune cells, such as macrophages (Reynolds, 1987). On the contrary, birds have less or even no phagocytic cells in healthy lavage samples. A small number of macrophages can be found on the epithelial lining of the parabronchi, whereas leucocytes are often present on the surface of the air sacs (Härtle and Kaspers, 2014). To

conclude, the avian intracellular distribution of GAM in PELF is difficult to measure, as macrophages are not a constitutively present cell population.

However, concentrations of FF in PELF could be measured after a single oral bolus of FF (Chapter 1.1).

MIC and PK/PD correlation

The sensitivity of *O. rhinotracheale* to antibiotics is very inconsistent and highly strain-dependent (Devriese, 1995; Devriese et al., 2001). In this study, a MIC_{50} of GAM against *O. rhinotracheale* of 2 µg/mL was obtained, while the MIC_{90} would be considered to be more than 32 µg/mL. This remarkable difference suggests an indication for acquired antimicrobial resistance of the pathogen against GAM. Also the MIC distribution of the 38 evaluated strains typically points towards acquired resistance as it has a bimodal distribution with 14 of the 38 isolates not belonging to the wild-type population (Fig. 3). Since GAM has never been used to treat an *O. rhinotracheale* infection, the acquired resistance might be a cross-resistance from other macrolides, such as erythromycin and tylosin. Devriese et al. (2001) evaluated the sensitivity and resistance to several macrolides in Belgian *O. rhinotracheale* strains. Tylosin also had a wide range of MIC values (1 to ≥ 64 µg/mL), although normal susceptibility levels for the type strain LMG 9086^T (MIC of ≤ 0.12 µg/mL) were observed. In the Netherlands, none of the tested *O. rhinotracheale* strains were inhibited by erythromycin or tilmicosin at a concentration of 64 µg/mL, whereas the MIC_{50} and MIC_{90} for tylosin were 4 and 8 µg/mL, respectively (van Veen et al., 2001). Also tylvalosin showed rather low MIC values against *O. rhinotracheale* strains originating from Germany and the Netherlands, with a MIC_{50} of 2 µg/mL and MIC_{90} of 8 µg/mL (Schwarz et al., 2012).

Since macrolides are classified as time-dependent antimicrobials with significant post-antibiotic effects, the efficacy of these antibiotics is generally accepted to correlate with both T>MIC and AUC_{last}/MIC (Van Bambeke and Tulkens, 2001; Andes et al., 2004; Hesje et al., 2007; Barbour et al., 2010; Giguère and Tessman, 2011). The established plasma cut-off values for Gram-negative bacteria are 40-50% for T>MIC, expressed for the dosage interval, and 125 for AUC/MIC, based on a 24 h period in plasma steady state conditions (Lees et al., 2008). As GAM has never been used to treat an infection with *O. rhinotracheale* resistant strains at the current dosage scheme, the authors decided to take only the susceptible population into account, in which the MIC_{90} was 2 µg/mL. Whether the T>MIC values

calculated in this study are high enough for a good efficacy of GAM against *O. rhinotracheale* is difficult to evaluate, as there are no cut-off values available for slowly eliminating antimicrobials, such as GAM. Recently, it was suggested that the time period to determine the T>MIC of these antibiotics may extend 24 h (Martinez et al., 2013).

Since GAM was given as a single bolus, no steady state situation was achieved, instead the AUC_{inf} was taken into account for plasma PK/PD correlations (Martinez et al., 2013). Furthermore, adapted cut-off values could be considered as lung tissue concentrations of macrolides tend to be higher than plasma concentrations. This was supported since a good efficacy for tulathromycin and azithromycin was correlated with low plasma AUC/MIC ratios, only 3.38 and 5, respectively (Lodise et al., 2005; Martinez et al., 2013). Similar values were found for GAM. Considering the AUC_{last}/MIC for lung tissue, the cut-off value of 125 can be accepted. Results far above and around this value were found for GAM. Both plasma AUC_{inf}/MIC and lung AUC_{last}/MIC results could therefore assume a good activity of GAM against *O. rhinotracheale*.

Conclusion

It can be concluded that the absorption of GAM after SC as well as PO administration is rapid and a high tissue distribution is reflected in the high V_d. Although the plasma concentrations after oral absorption are much lower than after SC injection, the lung concentrations after both routes of administration are nearly equivalent after 24 h. These high concentrations in the target lung tissue are of major importance for the treatment of respiratory infections, such as *O. rhinotracheale*. Nevertheless, an improved or appropriated formulation for oral therapy or an adjusted dose of GAM could improve the plasma and lung concentrations after PO administration.

The low GAM concentrations in PELF found in this study could be a result of the different anatomy of the respiratory system in birds compared to mammals, which would require a different collection method for PELF. Also a difference in immune cells present in the respiratory tract of birds compared to mammals might be responsible. To date, the collection of PELF in poultry is poorly investigated and requires more research. For macrolides, there is no single PK/PD index that correlates with efficacy for all members in

this class of antibiotics. The authors endeavor to correlate the plasma and lung PK parameters to the MIC values, but whether these values result in a therapeutic efficacy should be further determined in experimental and field infection studies (Marien et al., 2005, 2006, 2007; Garmyn et al., 2009a,b).

Acknowledgments

The authors would like to thank Veerle De Rop, Marleen Foubert and Arlette Van de Kerckhove for their laboratory assistance. For the aid in the animal experiment, we thank Gunther Antonissen, Nathan Broekaert, Thomas De Mil, Sophie Fraeyman, Elke Gasthuys and Charlotte Watteyn.

Chapter 2

Efficacy of selected antimicrobials in an *Ornithobacterium rhinotracheale* infection model in turkeys

Chapter 2.1

Efficacy of florfenicol against *Ornithobacterium rhinotracheale* in turkeys

Adapted from:

Watteyn A.[*], Russo E.[*], Garmyn A., De Baere S., Pasmans F., Martel A., Haesebrouck F., Montesissa C., De Backer P. and Croubels S. (2013). Clinical efficacy of florfenicol administered in the drinking water against *Ornithobacterium rhinotracheale* in turkeys housed at different environmental conditions: a PK/PD approach. *Avian Pathology*, 42(5), 474-481. [*] Shared first authorship

Abstract

In poultry rearing, medicated drinking water is a commonly used administration route, but drug uptake can be affected by many factors. In this study, the influence of two important parameters, photoperiod and feeding schemes, on florfenicol efficacy against *Ornithobacterium rhinotracheale* was evaluated. This Gram-negative bacterium is a respiratory pathogen that often affects turkeys.

All birds were oculonasally infected with *O. rhinotracheale* at a dose of 8.5 $\log_{10}$ cfu, preceded by infection with avian metapneumovirus (APV, at a dose of 4.4 $\log_{10}$ CD_{50}). The positive control group received no treatment. Florfenicol was given to the treated groups as a 5-day treatment of 30 mg/kg body weight florfenicol administered via drinking water and considering different photoperiods and feeding schemes (group 20/4L: photoperiod of 20 h, fed *ad libitum*; group 16/8L: photoperiod of 16 h, fed *ad libitum*; group 16/8R: photoperiod of 16 h, fed *ad libitum* but feed was withdrawn during the dark period and replaced 1 h after lighting). Starting from the APV infection till the end of the experiment, the animals were clinically examined and scored daily. Additionally, tracheal swabs were collected at several days post-bacterial infection (p.b.i.). Necropsy was performed at 6 and 14 days p.b.i. to evaluate the presence of gross lesions, and to collect trachea and lung tissue samples and airsac swabs for *O. rhinotracheale* quantification.

In all groups, a clinical improvement could be noticed, resulting in reduction of the clinical score. However, only the 16/8L and 16/8R groups showed significant differences from the control group. The results demonstrated an important influence of the photoperiod on the clinical outcome in an infection model. It can be advised that the photoperiod should be < 20 h to have sufficient drug intake. Nevertheless, there was no effect between fed and fasted turkeys for the clinical outcome.

Introduction

Viral and bacterial respiratory diseases often affect turkeys during the production round, resulting in economic losses due to an increased mortality and feed conversion rate, a reduction in growth rate and high medical costs (van Empel and Hafez, 1999).

Ornithobacterium rhinotracheale is a Gram-negative bacterium that affects the respiratory tract causing severe respiratory symptoms, depression, reduction in feed uptake and growth rate. To exert its pathogenic action, this bacterium needs the association with a predisposing factor that primarily affects the respiratory tract, like viral infections (avian metapneumovirus (APV), influenza virus, turkey rhinotracheitis virus, Newcastle disease virus) or environmental factors, such as poor management, inadequate ventilation, poor hygiene, high stocking density, high ammonia level, simultaneous infections, incorrect temperature and relative humidity, which can affect bird immunity (van Empel and Hafez, 1999).

To control *O. rhinotrachelae* infections in poultry-rearing, a strict biosecurity level and optimal environmental conditions are required. An effective vaccine is available but not commonly used in the field due to the seroselectivity (van Empel and Hafez, 1999; Murthy et al., 2007). Antimicrobial therapy can be applied during outbreaks but a careful evaluation has to be made on the antimicrobial agent choice as a high resistance level against a wide range of antimicrobial classes employed in aviculture has been reported (van Veen et al., 2001; Soriano et al., 2003; Zaini et al., 2008). This choice is hampered by the absence of a commercial screening method for the evaluation of antimicrobial sensitivity. A study evaluated the efficacy of three antimicrobial drugs in an *in vivo* infection model against *O. rhinotrachelae* in turkey, i.e. enrofloxacin (10 mg/kg), amoxicillin and florfenicol (FF, both 20 mg/kg), and enrofloxacin was found the most successful drug, followed by FF (Marien et al., 2006). However, in this research, plasma concentrations of FF were not measured and no correlation was made with the clinical outcome. Also no stability examinations of FF in the medicated drinking water were considered. A recent study proved the stability of FF during a ten day period at concentrations of 10 and 100 µg/mL (Muijsers et al., 2012).

Florfenicol is a broad spectrum synthetic antibiotic developed for veterinary use. It is a structure analogue of thiamphenicol (TAP), with a fluorine atom at the 3’ carbon position. This antibiotic acts as an inhibitor of the protein synthesis at the 50S ribosomal subunit by

blocking peptidyltransferase, and has a bacteriostatic action (Liu et al., 2003, Papich and Rivière, 2009). Efficacy of FF has been demonstrated against many animal diseases (Marien et al., 2007; Roiha et al., 2011; Thiry et al., 2011; Del Pozo Sacristan et al., 2012) and FF has been approved in Europe for treatment of fish, cattle, pigs and chickens (EMA, 2002). In pig and poultry farming it is current practice to administer antimicrobials via medicated feed or drinking water (Vermeulen et al., 2002). Drinking water medication is the most commonly used route of drug administration in intensively reared poultry, treating contemporaneously sick animals, but it can also give rise to some disadvantages. Drug intake between animals can vary dramatically due to both animal factors (hierarchy, flock size, sex, age, weight, species, breed, health status, etc.) and environmental factors (temperature, humidity, feed and water availability, photoperiod, etc.) (Vermeulen et al., 2002). This can lead to sub-bacteriostatic or –cidal concentrations, resulting in therapy failure.

The eating and drinking patterns alter depending the light scheme (Classen et al., 1994), which could have a huge influence on the uptake of drinking water medication. As FF is a time-dependent antibiotic (Hesje et al., 2007), it is important to have a frequent drug intake. Previously, the influence of the photoperiod was demonstrated on the pharmacokinetics and pharmacodynamics (PK/PD) (Watteyn et al., 2013). During a light period of 20 h the concentrations of FF showed a drop, while it was more constant during 16 h of light. Also different feeding schemes were investigated. However, no influence of feed restriction was observed. Accordingly, a study with the same different housing conditions based on photoperiod and feeding schemes is mandatory for the establishment of an efficient treatment protocol.

The aim of this research was to evaluate the efficacy of continuous water medication with 30 mg/kg body weight (BW) of FF during 5 days in turkeys against *O. rhinotrachelae* infection in an *in vivo* infection model.

Materials and methods

Micro-organisms

The *O. rhinotrachelae* strain LMG 9086^T was originally isolated from a turkey with a respiratory tract infection. The strain was serotyped as type A in an agar gel precipitation test, kindly performed by Prof. Hafez (Institute of Poultry Diseases, Free University of Berlin,

Germany; Hafez and Sting, 1999). The strain was cultured for 48 h at 37 °C on Columbia agar (Oxoid Ltd, Basingstoke, Hampshire, UK) with 5% sheep blood in a 5% CO_2 atmosphere, followed by growing into brain heart infusion broth (Oxoid) for 24 h at 37 °C with agitation. The cultured bacteria were washed twice in phosphate-buffered saline (PBS) followed each time by 5 min of centrifugation at 1509 *x g* at 4 °C. The bacterial challenge inoculum was prepared by resuspending the pellet in PBS to obtain a final concentration of 10^8 colony-forming units (cfu)/mL. To confirm the titre, 10-fold dilutions in PBS were inoculated on sheep blood agar and the number of colonies was counted.

The APV strain A/T6/96 (subtype A) was kindly donated by Prof. Nauwynck (Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Belgium). The strain was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande et al., 1998)

Veterinary drug

Florfenicol, 2,2-dichloro-N-[1S,2R)-1-(fluoromethyl)-2-hydroxy-2-[4-(methylsulfonyl)-phenyl]ethyl]-acetamide, was obtained from Zhejiang Hisoar Pharmaceutical Co., LTD (Zhejiang, China).

The medicated drinking water was prepared daily by stirring an appropriate solution for 30 minutes, followed by sonication for 20 minutes to dissolve the FF.

Clinical experiment

Fifty-six 1-day-old female turkeys (Moorgut Kartzfehn, Bösel, Germany) were housed according to the requirements of the European Union (Anonymous, 2010). They were kept together in an isolation room with HEPA-filtered air on wood shavings, had free access to feed and water, and received 15 h of light/day. At 3 weeks of age the animals were randomly divided in 4 groups (14 animals each) with different environmental conditions. The first group received 20 h light (between 8 h a.m. and 4 h a.m.)/4 h dark and was fed *ad libitum* (20/4L). The second group received 16 h light (between 8 h a.m. and 12 h p.m.)/8 h dark and was fed *ad libitum* (16/8L) and the third group had the same light cycle and was fed *ad libitum* except during the dark period in which feed was taken away (16/8R). These animals received feed again 1 h after the light was put on. The last group was a positive control group (C), with the same conditions as 16/8L.

All animals were negative for maternally derived antibodies to APV and *O. rhinotrachelae* at 2 weeks, by analysing the blood using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biochek, Gouda, the Netherlands). Tracheal swabs from all birds were collected and analysed to verify the absence of *O. rhinotrachelae*.

Table 1 displays the time schedule of the experiment.

At 23 days of age all animals were oculonasally infected with APV at a dose of 4.4 $\log_{10}$ ciliostatic dose (CD_{50}), using a virus stock titre of 5.5 $\log_{10}$ 50% CD_{50}/mL after the third passage in tracheal organ cultures.

Three days post-viral infection (p.v.i.) all animals were infected with *O. rhinotrachelae* at a dose of 8.5 $\log_{10}$ cfu by dividing a total of 250 µL of inoculum equally over the nostrils and eyes.

The control group (C) was not treated with FF, while the 3 other groups (20/4L, 16/8L and 16/8R) received FF continuously via the drinking water during a 5-day period (target dose: 30 mg/kg BW/day) starting one day post-bacterial inoculation (p.b.i.). Animals were all weighed the day of APV inoculation, and again the day of *O. rhinotrachelae* inoculation. The water uptake was measured daily from 3 days before until the end of the treatment. These data were used to determine the inclusion of FF in the medicated drinking water and to evaluate the exact amount of drug daily received (based on BW and drinking water intake).

A clinical examination of all turkeys was made daily until 14 days p.b.i., and the clinical signs of animals were scored as follows: 0, no clinical signs; 1, clear nasal exudates; 2, turbid nasal exudates; 3, nasal exudates with mild swollen infra-orbital sinuses; 4, nasal exudates with extreme swollen infra-orbital sinuses; 5, nasal exudates with extreme swollen infra-orbital sinuses and frothy eyes; 6, death.

Tracheal swabs were collected from all groups for quantification of *O. rhinotrachelae* using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc., Corona, USA) at 1, 2, 4, 6, 8, 10 and 14 days p.b.i. Swabs were processed immediately after collection and the quantification (cfu/mg of mucus) was performed as described by Marien et al. (2005).

Six birds of each group were sacrificed at 6 days p.b.i. and the remaining birds were sacrificed at 14 days p.b.i. Euthanasia was performed by intravenous injection of 0.3 mL/kg BW of T61 (Intervet, Belgium). Necropsy of all birds was performed to evaluate the presence of gross lesions. At 6 days p.b.i., samples of the trachea and lungs were collected for *O. rhinotrachelae* quantification. A 10% tissue suspension in PBS was prepared from these

samples. The air sacs were sampled with cotton swabs for bacterial isolation. At day 14 p.b.i. the trachea, lungs and air sacs were sampled with cotton swabs for *O. rhinotrachelae* isolation. All samples were processed immediately after collection following the procedure described by Garmyn et al. (2009a,b).

The clinical trial was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2011/001).

Table 1. Time schedule of the infection experiment.

Day	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Infection	APV			ORT														
Therapy					X	X	X	X	X									
Clinical score	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Tracheal swabs					X	X		X		X		X		X				X
Necropsy										n=6								n=8

APV, avian metapneumovirus; ORT, *O. rhinotracheale*

Statistical analyses

The parameters were statistically analysed by means of single-factor analysis of variance (ANOVA) with Bonferroni correction. The area under the curve ($AUC_{day\ 1\text{-}6pbi}$) of the bacterial titre and the isolation of *O. rhinotrachelae* in trachea and lung were analysed by the Kruskal Wallis test. A P-value < 0.05 was considered statistically significant.

For the analysis of clinical score and tracheal swabs, both the mean score and the AUC of the clinical score-time curve and the titre-time curve, respectively, were considered. The AUC was calculated by the linear trapezoidal rule. These statistical analyses were performed using SPSS Statistics 20 (IBM SPSS software, New York, USA).

Results

During the experiments, mortalities did not take place in any of the experimental groups.

Tracheal swabs before infection were all negative for *O. rhinotrachelae*.

Mean BW (± SD) for each group of turkeys at the day before the bacterial infection, 6 and 14 days p.b.i. are reported in Table 2. At all occasions, there was no significant difference in BW between groups.

As can be seen in Figure 1, respiratory signs were observed in the four experimental groups, starting from day 1 p.b.i., followed by a decrease of the clinical score around day 4 p.b.i. There were statistically significant differences between 16/8L vs. C (17.33 ± 5.04 and 34.00 ± 8.53, respectively, with $p<0.01$) and 16/8R vs. C (19.86 ± 6.34 and 34.00 ± 8.53, respectively, with $p<0.01$) for $AUC_{day\ 1\text{-}14\ pbi}$ (mean ± SD) of the clinical score.

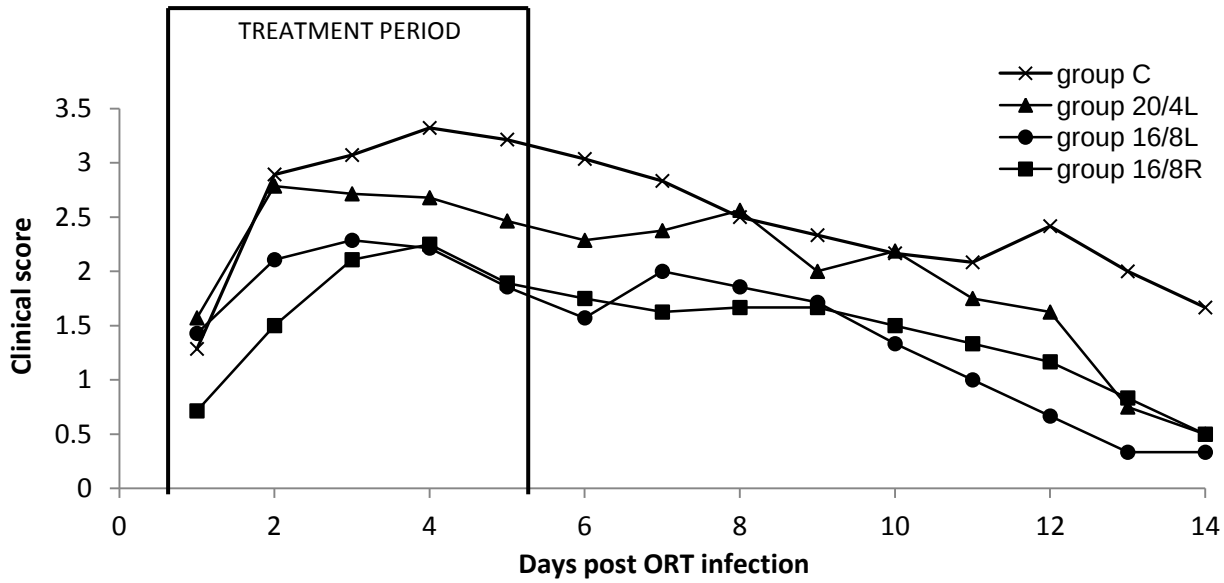

Figure 1. Mean clinical scores of turkeys inoculated with avian metapneumovirus and *O. rhinotracheale*, and which were not treated (group C, ×) or treated with 30 mg/kg of florfenicol via medicated water for 5 days: group 20/4L (20h light and 4 h dark, fed *ad libitum*, ▲), group 16/8L (16 h light and 8 h dark, fed *ad libitum*, ●), group 16/8R (16 h light and 8 h dark, fed *ad libitum* from 1h after the lighting, ■).

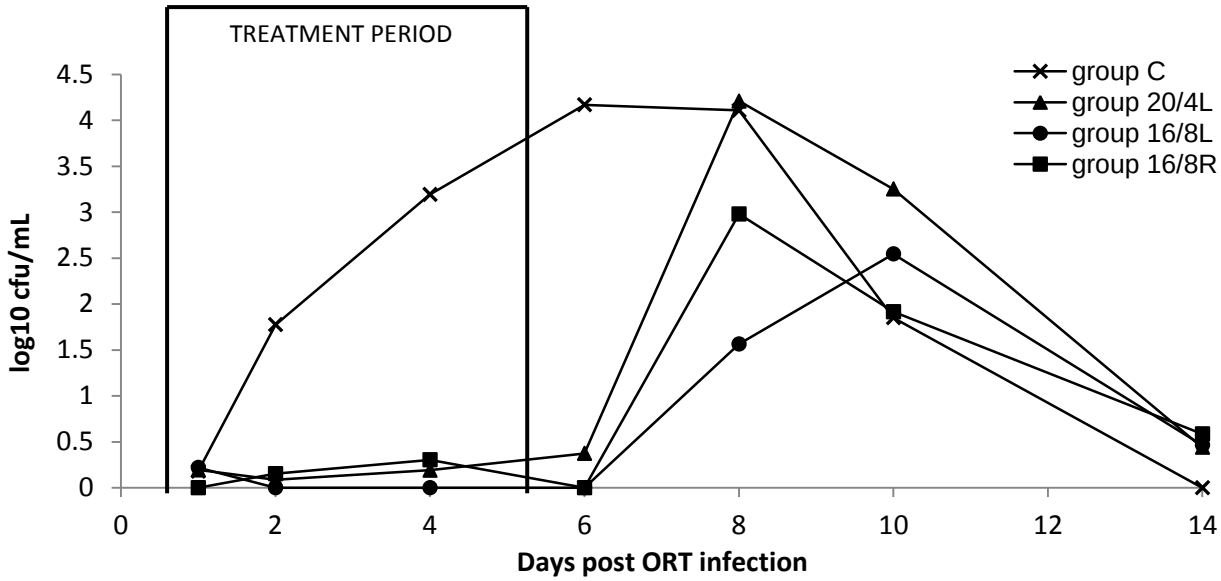

Figure 2. Mean *O. rhinotracheale* titres in tracheal swabs collected from turkeys inoculated with avian metapneumovirus and *O. rhinotracheale*, and which were not treated (group C, ×) or treated with 30 mg/kg of florfenicol via medicated water for 5 days: group 20/4L (20h light and 4 h dark, fed *ad libitum*, ▲), group 16/8L (16 h light and 8 h dark, fed *ad libitum*, ●), group 16/8R (16 h light and 8 h dark, fed *ad libitum* from 1h after the lighting, ■).

Figure 2 shows *O. rhinotrachelae* titres in tracheal swabs over a period of 14 days. Up to 6 days p.b.i. no increase in titres could be observed in the groups treated with FF. This resulted in significant differences for the $AUC_{day\ 1\text{-}14\ pbi}$ (mean ± SD) between C (30.06 ± 5.28) and all treated groups (17.06 ± 9.62, 14.13 ± 5.59, 11.30 ± 8.06, with $p<0.05$, $p<0.01$ and $p<0.01$ for 20/4L, 16/8L and 16/8R respectively).

Results of necropsy are depicted in Table 2. Bacterial titres of trachea and lung samples collected 6 days p.b.i. showed a significant difference between the non-treated group (group C) and all other groups. Samples collected 14 days p.b.i. showed no statistical difference between groups. Almost all tissues were negative for *O. rhinotrachelae*.

Table 2. Clinical score, tracheal *O. rhinotrachele* titres, isolation of *O. rhinotrachele* from several organs and mean body weight of turkeys inoculated with avian metapneumovirus and *O. rhinotrachele* with an interval of 3 days and treated with 30 mg/kg FF via drinking water for 5 days, with different photoperiods.

Group	Clinical score		*O. rhinotracheale* titres in tracheal swabs		Isolation of *O. rhinotracheale*						Body weight (g)		
					6 days pbi (log10cfu/mL)			14 days pbi (n positive/n tested)			Before infection	6 days pbi	14 days pbi
	AUC day 1-6 pbi	AUC day 1-14 pbi	AUC day 1-6 pbi	AUC day 1-14 pbi	Trachea	Lung	Airsac	Trachea	Lung	Airsac			
C	14.7 ± 5.5 ^{A}	34.0 ± 8.5 ^{A}	13.3 ± 2.2 ^{A}	30.1 ± 5.3 ^{A}	6/6 6.01 ± 0.20 ^{A}	6/6 3.63 ± 0.69 ^{A}	2/6	0/8	0/8	0/8	442.1 ± 69.5 ^{A}	702.1 ± 102.8 ^{A}	1146.0 ± 171.1 ^{A}
20/4L	12.6 ± 4.2 ^{AB}	27.9 ± 6.6 ^{AB}	0.98 ± 2.7 ^{B}	17.1 ± 9.6 ^{B}	2/6 0.88 ± 1.36 ^{B}	1/6 0.65 ± 1.59 ^{B}	0/6	1/8	0/8	0/8	429.3 ± 60.0 ^{A}	775.6 ± 104.9 ^{A}	1240.0 ± 147.5 ^{A}
16/8L	9.0 ± 2.6 ^{B}	17.3 ± 5.0 ^{C}	0.84 ± 1.7 ^{B}	14.1 ± 5.6 ^{B}	0/6 0.00 ^{B}	0/6 0.00 ^{B}	0/6	3/8	0/8	0/8	433.2 ± 60.3 ^{A}	763.4 ± 101.6 ^{A}	1095.5 ± 142.0 ^{A}
16/8R	10.0 ± 2.5 ^{B}	19.9 ± 6.3 ^{BC}	0.11 ± 0.4 ^{B}	11.3 ± 8.1 ^{B}	0/6 0.00 ^{B}	0/6 0.00 ^{B}	0/6	1/8	0/8	0/8	450.7 ± 50.4 ^{A}	779.9 ± 112.9 ^{A}	1205.7 ± 149.8 ^{A}

All results are presented as mean ± SD.

Group 20/4L, 20h light and 4 h dark, fed *ad libitum*; group 16/8L, 16 h light and 8 h dark, fed *ad libitum*; group 16/8R, 16 h light and 8 h dark, fed *ad libitum* from 1 h after the lighting, all treated with FF (30 mg/kg BW); or no treatment (group C). Data are presented as the mean value ± standard deviation.

$^{A, B, C, D}$ Treatments sharing a letter do not differ from one another at the 5% global significance level.

Discussion

Medicated drinking water is a commonly used administration route to treat intensively reared poultry. As already reported, drug intake however can be affected by many factors using this way of oral medication. In this study, the influence of two important parameters, namely photoperiod and feeding schemes on FF uptake using drinking water administration, has been tested. The results demonstrated an important influence of the photoperiod on the clinical outcome in an ORT infection model. Nevertheless, there was no effect of the feeding schemes.

The clinical scores highlight a difference between the two photoperiods, the 20/4L group has a scoring that is intermediate between the other two treated groups and the untreated control group. The replication degree of *O. rhinotrachelae* in the trachea at the end of the therapy confirms this difference. This is in accordance with our PK/PD findings previously discussed (Watteyn et al., 2013). The decreased efficacy of the therapy in the group 20/4L is most probably related to the irregular and too low plasma concentration of the drug as mentioned in the PK/PD study. Indeed, the PK/PD index T>MIC was below 40% in the group 20/4L, suggesting an insufficient concentration of FF to be efficacious to eradicate the bacterium (Watteyn et al., 2013).

No differences were seen between the groups 16/8L and 16/8R. Therefore, we can conclude that there was no effect of feed restriction on the therapeutic outcome.

Marien et al. (2006) tested a commercially available drinking water formulation of FF (20 mg/kg BW) in a similar *in vivo* infection mode and reported no significant reduction in clinical symptoms. FF did reduce the bacterial titre, but only to 3 $\log_{10}$ cfu/g mucus. However, during our clinical trial, the administration of a higher dose of 30 mg FF/kg BW *via* the drinking water for a 5-day period was able to reduce the bacterial titre in tracheal swabs at 6 days p.b.i. to less than 1 $\log_{10}$ cfu/mL in all treated groups. This discrepancy between the two studies could be related to the different dose of the drug.

For every treated group, the bacterial titre remained below 0.5 $\log_{10}$ cfu/mL during the administration of the drug. A few days after the end of the treatment, there was an increase of the bacterial growth to 3 and 4 $\log_{10}$ cfu/mL for the groups with 16 and 20 h of light, respectively. This is in accordance with the PK/PD findings reported by Watteyn et al.

(2013b). Florfenicol was eliminated out of the body after 24 h. However, the mean clinical score in the first 3 days of antibiotic administration was high, in which the control group had the highest score, followed by respectively the groups with 20 h and 16 h of light. The presence of the clinical symptoms in the treated groups seems to be rather associated with APV infection alone as already reported by other investigators (Van de Zande et al., 2001). From day 4 of treatment onward, these clinical scores were strongly reduced. Accordingly, it can be stated that the administration of FF at the onset of the viral respiratory infection can significantly reduce clinical symptoms caused by secondary bacterial infections, such as *O. rhinotrachelae*.

In conclusion, this study confirms the need for a dark period of more than 4 h in medicated drinking water administration. The continuous administration of 30 mg of FF/kg BW *via* the drinking water for 5 days at a photoperiod of 16 h seems to be effective to cure of an *O. rhinotracheale* infection in turkeys.

Acknowledgements

The authors wish to thank Arlette Van de Kerckhove and Anja Van den Bussche for their excellent technical assistance, and Mathias Devreese, Joline Goossens, Jelle Lambrecht and Elke Plessers for their aid in the animal experiment.

Chapter 2.2

Efficacy of gamithromycin against *Ornithobacterium rhinotracheale* in turkeys

Adapted from:

Watteyn A., Devreese M., Plessers E., Wyns H., Garmyn A., Reddy V., Pasmans F., Martel A., Haesebrouck F., De Backer P. and Croubels S. Efficacy of gamithromycin in an *Ornithobacterium rhinotracheale* challenge model in turkeys. *Avian Pathology* (Conditionally accepted).

Abstract

Ornithobacterium rhinotracheale is a common avian respiratory pathogen that often affects turkeys during the production round, resulting in important economic losses. The objective of this study was to evaluate the clinical efficacy of gamithromycin (GAM) against *O. rhinotracheale* in turkeys. The birds were oculonasally inoculated with 8.5 $\log_{10}$ colony forming units of *O. rhinotracheale*, preceded by infection with avian metapneumovirus (APV, at 4.4 $\log_{10}$ 50% ciliostatic dose). Besides a negative (CONTR-, not infected, not treated) and a positive control group (CONTR+, infected, not treated), there were two treated groups administered GAM (6 mg/kg body weight) either subcutaneously (GAM SC) or orally (GAM PO) as a single bolus at one day post-bacterial infection (p.b.i.). Starting from the APV infection till the end of the experiment, the animals were clinically examined and scored daily. Additionally, tracheal swabs were collected at several days p.b.i. Necropsy was performed at 4, 8 and 12 days p.b.i. to evaluate the presence of gross lesions, and to collect trachea and lung tissue samples and airsac swabs for *O. rhinotracheale* quantification.

The clinical score of the GAM SC group showed slightly lower values and birds recovered earlier compared to GAM PO and CONTR+. *O. rhinotracheale* titres were significantly reduced in tracheal swabs of the SC group between 2 and 4 days p.b.i. At necropsy, CONTR+ showed higher *O. rhinotracheale* titres and lung tissues compared to the treated groups. Moreover, at 8 days p.b.i. only the lung samples of CONTR+ were positive.

In conclusion, the efficacy of GAM against *O. rhinotracheale* was demonstrated in the lung tissue. However, the PO bolus administration of the commercially available product was not as efficacious as the SC bolus.

Introduction

Ornithobacterium rhinotracheale is a Gram-negative bacterium, which causes respiratory disease in poultry, characterized by pneumonia, tracheitis and airsacculitis (Hinz et al., 1994). An infection with this agent often results in severe economic losses due to increased mortality and feed conversion rate, reduction in growth rate and egg production as well as increased medical costs (Van Empel and Hafez, 1999). In Canada, *O. rhinotracheale* is even the third most frequently diagnosed pathogen in turkeys (Agunos et al., 2013). A bacterin vaccine against this bacterium is available, but not commonly used in the field, as it does not provide strong and cross-protection against the various serotypes of *O. rhinotracheale* (Van Empel and Hafez, 1999; Schuijffel et al., 2006; Murthy et al., 2007). Hence, antimicrobial drug therapy is most frequently applied, but the sensitivity of *O. rhinotracheale* to antibiotics is strain-dependent (Devriese, 1995; Devriese et al., 2001; Schwarz et al., 2012; Watteyn et al., 2015). The efficacy of drinking water therapy has already been tested for different antimicrobial drugs using an *O. rhinotracheale* infection model in turkeys, and enrofloxacine as well as florfenicol were found to be effective to cure the infection (Marien et al., 2006; Watteyn et al., 2013b), whereas amoxicillin was not (Marien et al., 2006). Moreover, the photoperiod has an important influence on the water consumption and consequently on the uptake of drugs. Accordingly, this may have an effect on the pharmacokinetics (PK) of drugs when applied in drinking water, as well as on the clinical outcome (Watteyn et al., 2013b). The latter study in turkeys demonstrated that the photoperiod should preferably be less than 20 h to have sufficient florfenciol (FF) intake.

Macrolides are frequently used antibiotics in veterinary medicine. Their mode of action is based on inhibition of bacterial protein biosynthesis at the 23S ribosomal RNA in the 50S subunit of the ribosome, where they bind to different bases of the peptidyl transferase centre and prevent the translocation process (Cobos-Trigueros et al., 2009). Gamithromycin (GAM) is a new macrolide and member of the azalide class. At present, it is only registered for use in cattle to treat Bovine Respiratory Disease (BRD). GAM is a long-acting antibiotic with accumulation in lung tissue as supported by its extended elimination half-life and large volume of distribution in several animal species, including turkeys (Huang et al., 2010; Giguère et al., 2011; Watteyn et al., 2013a; Wyns et al., 2014; Watteyn et al., 2015).

To date, no studies have been reported demonstrating the efficacy of any macrolides in an *in vivo O. rhinotracheale* infection model in turkeys. The remarkable PK and pharmacodynamic (PD) properties of GAM are interesting to treat turkeys of an *O. rhinotracheale* infection. Therefore, the aim of this study was to examine the efficacy of GAM in a dual infection model with APV/*O. rhinotracheale* in turkeys. This was examined on basis of clinical signs, titration of the bacterium and body weight (BW) gain. Necropsy was performed at different time points to evaluate the long-acting properties of GAM. Apart from a single subcutaneous (SC) administration, also a single oral (PO) administration was assessed, both at a dose of 6 mg/kg BW.

Materials and methods

Micro-organisms

The *O. rhinotracheale* strain LMG 9086^T was originally isolated from a turkey with a respiratory tract infection. The minimum inhibitory concentration (MIC) of GAM against this strain is 0.5 µg/mL (Watteyn et al., 2015). LMG 9086^T was serotyped as type A in an agar gel precipitation test, kindly performed by Prof. Hafez (Institute of Poultry Diseases, Free University of Berlin, Germany; Hafez and Sting, 1999). The strain was cultured for 48 h at 37 °C on Columbia agar (Oxoid Ltd, Basingstoke, Hampshire, UK) with 5% sheep blood in a 5% CO_2 atmosphere, followed by growing into brain heart infusion broth (Oxoid) for 24 h at 37 °C with agitation. The cultured bacteria were washed twice in phosphate-buffered saline (PBS) followed each time by 5 min of centrifugation at 1509 *x g* at 4 °C. The bacterial challenge inoculum was prepared by resuspending the pellet in PBS to obtain a final concentration of 10^8 colony forming units (cfu)/mL. To confirm the titre, 10-fold dilutions in PBS were inoculated on sheep blood agar and the number of colonies was counted.

The used APV strain A/T6/96 (subtype A) was isolated during a respiratory outbreak on a Belgian turkey farm (Van de Zande et al., 1998). The virus stock had a titre of 5.3 $\log_{10}$ 50% ciliostatic dose (CD_{50})/mL after the third passage in tracheal organ cultures.

Veterinary drug

The veterinary drug Zactran (Merial Ltd, North Brunswick, NJ, USA) contains 150 mg/mL (15.0% w/v) GAM as active substance in a buffered solution with monothioglycerol, succinic

acid and glycerol formal. This commercially available formulation was diluted with *aqua ad injectabilia* up to a concentration of 15 mg GAM/mL (1.5% weight/volume). This solution was used for both SC injection and PO dosing.

Animal experiment

The animal experiment was based on an avian metapneumovirus (APV) – *O. rhinotracheale* challenge infection model described by Marien et al. (2005). Sixty-four one-day-old non-vaccinated female turkeys (Moorgut Kartzfehn, Bösel, Germany) were housed according to European (Anonymous 2010). They were housed in group in an isolation room with HEPA-filtered air on wood shavings, had free access to feed and water, and the light schedule was 16 h of light/day. At 18 days of age, they were randomly divided in four groups (16 animals each), a negative control group (CONTR-; not infected, not treated), a positive control group (CONTR+; infected, not treated) and two treated groups (GAM SC and GAM PO, both infected and treated), and each group was housed separately.

All animals were negative for maternally derived antibodies to APV and *O. rhinotracheale* at 2 weeks of age, as tested with enzyme-linked immunosorbent assays (ELISA). Additionally, tracheal swabs (Copan Diagnostics Inc., Corona, USA) from all birds were collected one day before *O. rhinotracheale* inoculation and analysed to verify the absence of *O. rhinotracheale*. Table 1 displays the time schedule of the experiment.

At 22 days of age all animals, except the CONTR- group, were inoculated with 4.4 $\log_{10}$ CD_{50} APV, by dividing a total volume of 250 µL of inoculum equally over the nostrils and eyes. Three days post-viral infection (p.v.i.), all animals were oculonasally inoculated with 8.5 $\log_{10}$ cfu *O. rhinotracheale* as described above. The turkeys in the CONTR- group received an equal volume of PBS in the nostrils and eyes.

The turkeys in the treated groups received at one day post-bacterial inoculation (p.b.i.) a single bolus of GAM (6 mg/kg BW) either SC in the neck region (GAM SC) or PO in the crop (GAM PO).

All animals were weighed at the day of *O. rhinotracheale* inoculation and at the day of necropsy.

A clinical examination of all turkeys was performed daily until 12 days p.b.i., and the clinical signs were scored as follows: 0, no clinical signs; 1, clear nasal exudates; 2, turbid nasal exudates; 3, nasal exudates with mild swollen infra-orbital sinuses; 4, nasal exudates with

extreme swollen infra-orbital sinuses; 5, nasal exudates with extreme swollen infra-orbital sinuses and frothy eyes; 6, death.

Tracheal swabs were collected from all birds for quantification of *O. rhinotracheale* using cotton-tipped aluminium shafted swabs (Copan Diagnostics Inc., Corona, USA) at 1, 2, 4, 6, 8, 10 and 12 days p.b.i. Swabs were processed immediately after collection and the number of cfu/mL was determined as described by Marien et al. (2005).

Six birds of each group were sacrificed at 4 and 8 days p.b.i., and the remaining four birds were sacrificed at 12 days p.b.i. The animals were intramuscularly sedated with a combination of xylazine (XylM 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100, Virbac, Wavre, Belgium), followed by exsanguination.

Necropsy of all birds was performed to evaluate the presence of gross lesions. Tracheal tissue, sampled from larynx to syrinx, and the entire lung were collected for *O. rhinotracheale* quantification. A 10% (w/v) tissue suspension in PBS was prepared from these samples. The thoracal air sacs were sampled for bacterial isolation using cotton swabs. All samples were processed immediately after collection following the procedure described by Garmyn et al. (2009a,b).

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC2013/182).

Table 1. Time schedule of the infection experiment.

Day	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12
Infection	APV			ORT												
Therapy					X											
Clinical score	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Tracheal swabs			X		X	X		X		X		X		X		
Necropsy								n=6				n=6				n=4

APV, avian metapneumovirus; ORT, *O. rhinotracheale*

Statistical analyses

The following parameters were statistically analysed by means of single-factor analysis of variance (ANOVA) with Bonferroni correction: body weight, the area under the curve of the clinical score from day 2 till day 12 p.b.i. ($AUC_{day\ 2\ to\ 12\ p.b.i.}$) and *O. rhinotracheale* titres in tracheal swabs. The $AUC_{day\ 2\ to\ 4\ p.b.i.}$, $AUC_{day\ 2\ to\ 8\ p.b.i.}$, both for the clinical score and *O. rhinotracheale* titres in the tracheal swabs, and the titres of *O. rhinotracheale* in trachea and lung tissue on day 4, 8 and 12 were analysed by the Kruskal Wallis test, as these parameters were not homogenous. A *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 22 (IBM, New York, USA).

Results

Body Weight

The mean BW (± SD) for the different groups at several time points are listed in Table 2. Only at 8 days p.b.i., the CONTR- had a significant higher BW compared to the infected groups.

Clinical signs

Mortalities did not occur in any of the experimental groups. The negative control group showed no clinical signs and was at all time points negative for *O. rhinotracheale*. All the infected groups had similar mean clinical scores, but GAM SC fully recovered three days earlier than the other two groups (Figure 1). As indicated in Table 2, the AUC of the clinical scoring from day 0 to the day of necropsy (day 4, 8 or 12) showed no significant difference between the infected groups (Table 2). By the last day of the experiment, the remaining turkeys of all groups clinically recovered from the APV/*O. rhinotracheale* infection.

Macroscopic findings

At necropsy, no gross lesions were found in any of the birds.

Bacterial titration of tracheal swabs

The tracheal swabs, collected one day before *O. rhinotracheale* inoculation were all negative for *O. rhinotracheale*. The bacterial titration of the tracheal swabs were compared between groups by means of the AUC from day 0 to day of necropsy (Table 2). The $AUC_{day\ 2\ to\ 4\ p.b.i.}$ of

GAM SC was significantly lower than CONTR+. The other results showed no significant differences. As can be seen in Figure 2, the tracheal swabs were positive for *O. rhinotracheale* till day 6 p.b.i. for the CONTR+ group, whereas for GAM SC and GAM PO they were positive till day 8 and 10 p.b.i., respectively.

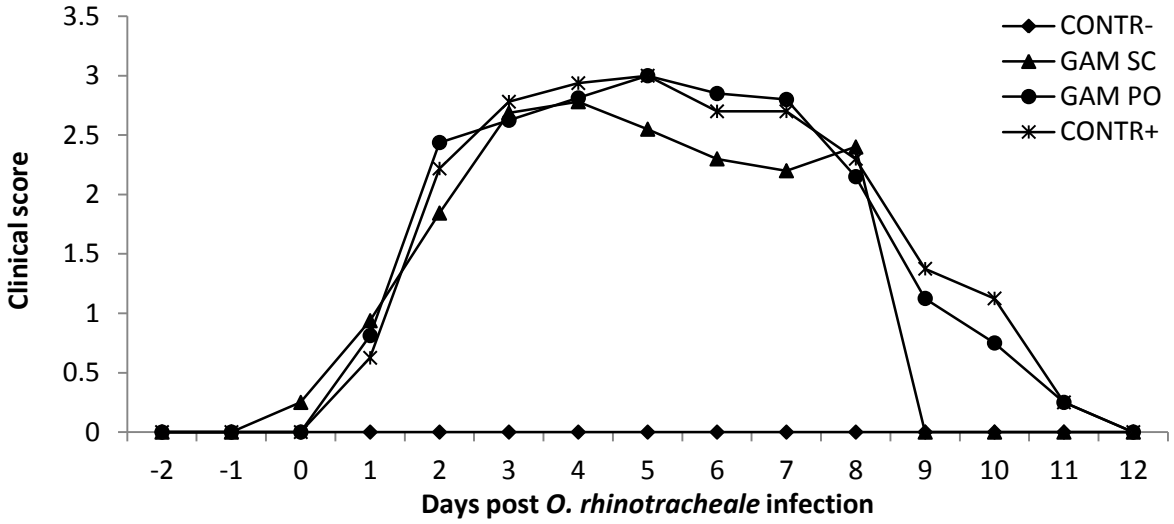

Figure 1. Mean clinical score of turkeys inoculated with APV and *O. rhinotracheale*, and which were not treated (CONTR+), or treated with a single dose of 6 mg/kg body weight gamithromycin, either subcutaneously (GAM SC) or orally (GAM PO). Turkeys from the CONTR- group were not infected and not treated.

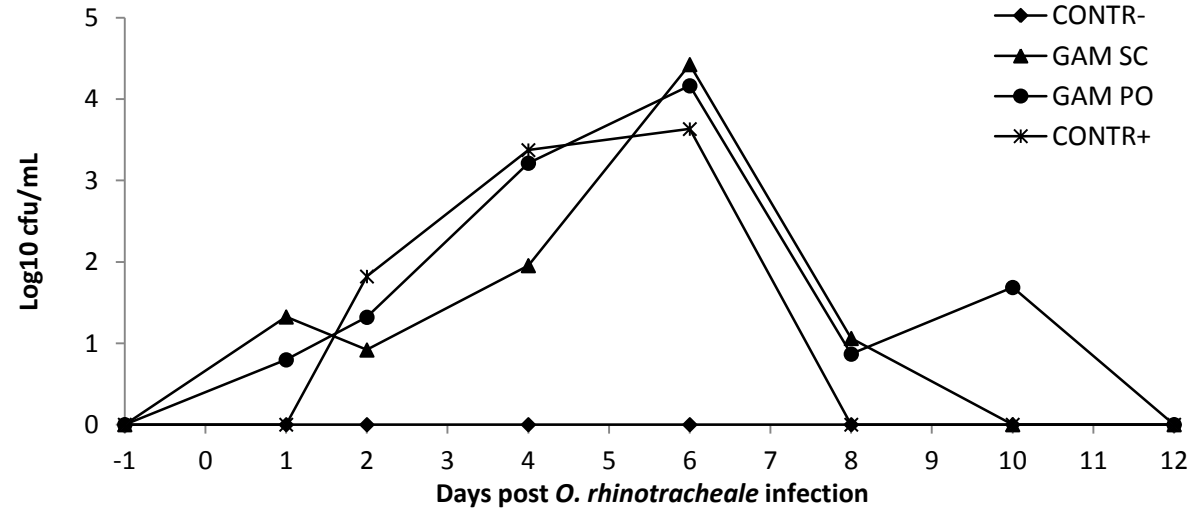

Figure 2. Mean *O. rhinotracheale* titres in tracheal swabs collected from turkeys inoculated with APV and *O. rhinotracheale*, and which were not treated (CONTR+), or treated with a single dose of 6 mg/kg body weight gamithromycin, either subcutaneously (GAM SC) or orally (GAM PO). Turkeys from the CONTR- group were not infected and not treated.

Table 2. Mean ± SD body weight, clinical score and tracheal *O. rhinotracheale* titres of turkeys inoculated with APV and *O. rhinotracheale* with an interval of 3 days, treated once with 6 mg/kg body weight gamithromycin (GAM) subcutaneously (SC) or orally (PO) at 1 day p.b.i.

Group	CONTR-	GAM SC	GAM PO	CONTR+
Body weight (kg)				
Before infection	0.81 ± 0.08^{A}	0.80 ± 0.08^{A}	0.77 ± 0.09^{A}	0.74 ± 0.08^{A}
4 days p.b.i.	0.97 ± 0.11^{A}	0.96 ± 0.10^{A}	0.99 ± 0.08^{A}	0.94 ± 0.15^{A}
8 days p.b.i.	1.40 ± 0.09^{A}	1.18 ± 0.11^{B}	1.17 ± 0.14^{B}	1.18 ± 0.12^{B}
12 days p.b.i.	1.40 ± 0.37^{A}	1.66 ± 0.20^{A}	1.46 ± 0.15^{A}	1.50 ± 0.05^{A}
Clinical score				
$AUC_{day\ 2\ to\ 4\ p.b.i.}$	0.0 ± 0.0^{A}	5.14 ± 1.04^{B}	5.25 ± 1.21^{B}	5.36 ± 1.51^{B}
$AUC_{day\ 2\ to\ 8\ p.b.i.}$	0.0 ± 0.0^{A}	14.45 ± 2.14^{B}	15.60 ± 3.00^{B}	16.33 ± 5.99^{B}
$AUC_{day\ 2\ to\ 12\ p.b.i.}$	0.0 ± 0.0^{A}	17.25 ± 1.90^{B}	18.81 ± 5.38^{B}	17.50 ± 7.34^{B}
O. rhinotracheale titres in tracheal swabs				
$AUC_{day\ 2\ to\ 4\ p.b.i.}$	0.0 ± 0.0^{A}	2.87 ± 2.82^{B}	4.53 ± 2.47^{BC}	5.20 ± 2.88^{C}
$AUC_{day\ 2\ to\ 8\ p.b.i.}$	0.0 ± 0.0^{A}	13.93 ± 9.31^{B}	16.75 ± 5.88^{B}	14.11 ± 8.27^{B}
$AUC_{day\ 2\ to\ 12\ p.b.i.}$	0.0 ± 0.0^{A}	21.31 ± 7.83^{B}	23.73 ± 9.07^{B}	13.19 ± 7.36^{AB}

CONTR-, not infected and not treated; GAM SC: infected and treated with GAM subcutaneously; GAMI PO, infected and treated with GAM orally; CONTR+, infected and not treated

Data presented as mean ± SD. Groups sharing an uppercase superscript letter within one row do not differ from one another at the 5% global significance level.

p.b.i., post bacterial infection

Table 3. Isolation of *O. rhinotracheale* from trachea, lung and airsac samples, expressed as mean ± SD log_{10} CFU/mL and number of positive samples/number tested samples, in turkeys inoculated with APV and *O. rhinotracheale* with an interval of 3 days, treated once with 6 mg/kg body weight gamithromycin (GAM) subcutaneously (SC) or orally (PO) at 1 day p.b.i.

Group	4 days p.b.i.			8 days p.b.i.			12 days p.b.i.		
	Trachea	Lung	Airsac	Trachea	Lung	Airsac	Trachea	Lung	Airsac
CONTR-	0.0 ± 0.0 ^{A} 0/6	0.0 ± 0.0 ^{A} 0/6	0/6	0.0 ± 0.0 ^{A} 0/6	0.0 ± 0.0 ^{A} 0/6	0/6	0.0 ± 0.0 ^{A} 0/4	0.0 ± 0.0 ^{A} 0/4	0/4
GAM SC	2.08 ± 1.69 ^{AB} 4/6	1.04 ± 1.71 ^{AB} 2/6	2/6	1.28 ± 3.14 ^{A} 1/6	0.0 ± 0.0 ^{A} 0/6	0/6	0.0 ± 0.0 ^{A} 0/4	0.0 ± 0.0 ^{A} 0/4	0/4
GAM PO	2.37 ± 1.19 ^{AB} 5/6	2.21 ± 1.17 ^{BC} 5/6	2/6	0.0 ± 0.0 ^{A} 0/6	0.0 ± 0.0 ^{A} 0/6	0/6	0.0 ± 0.0 ^{A} 0/4	0.0 ± 0.0 ^{A} 0/4	0/4
CONTR+	4.23 ± 2.20 ^{B} 5/6	3.27 ± 1.64 ^{C} 5/6	2/6	0.42 ± 1.02 ^{A} 1/6	1.20 ± 1.32 ^{B} 3/6	0/6	0.0 ± 0.0 ^{A} 0/4	0.0 ± 0.0 ^{A} 0/4	0/4

CONTR-, not infected and not treated; GAM SC: infected and treated with GAM subcutaneously; GAMI PO, infected and treated with GAM orally; CONTR+, infected and not treated;

Data presented as mean ± SD. Groups sharing an uppercase superscript letter within one column do not differ from one another at the 5% global significance level.

p.b.i., post bacterial infection

Bacterial titration in tissue samples

The results of the isolation of *O. rhinotracheale* from the necropsy samples are shown in Table 3. At 4 days p.b.i., 4/6, 5/6 and 5/6 of the trachea samples were positive for *O. rhinotracheale* in the GAM SC, GAM PO and CONTR+ groups, respectively. The mean bacterial titres of these samples were 2.08 and 2.37 $\log_{10}$ cfu/mL for GAM SC and GAM PO groups, respectively, whereas the titre of the CONTR+ group was about 2 $\log_{10}$ higher (4.23 $\log_{10}$ cfu/mL). The *O. rhinotracheale* titres of the lung samples at 4 days p.b.i. were significantly lower for the GAM SC group compared to CONTR+. For all infected groups, two out of six airsac samples were positive. At 8 days p.b.i., only the lung samples of the CONTR+ group were positive for *O. rhinotracheale*, resulting in a significant difference with the GAM SC and GAM PO groups. All samples were negative at 12 days p.b.i.

Discussion

This is the first study investigating the *in vivo* efficacy of a macrolide antibiotic against *O. rhinotracheale* infections in turkeys.

The commercial formulation of GAM is registered for SC use in cattle only. Accordingly, GAM was also administered SC in this study. On the other hand, as mass medication through drinking water is the most important route of drug administration in poultry, the same diluted formulation of GAM was also given orally as a single bolus in the crop at the same dose. An earlier study demonstrated different plasma PK properties of GAM between both routes of administration in turkeys, mainly with respect to the bioavailability. There is a lower absorption of the antimicrobial after PO administration compared to SC, with a relative bioavailability of GAM after PO administration of only 25% (Watteyn et al., 2015). In the current study, a slightly improved clinical recovery was observed after SC administration of GAM, compared to the PO administration. This can be explained by the PK parameters in lung tissue, since a difference in mean maximum concentration obtained after single SC and PO administration of the same dose was seen in lung tissue, i.e. 3.66 and 2.22 µg/g after SC and PO administration, respectively (Watteyn et al., 2015). Moreover, the mean elimination half-life of GAM in lung tissue was 92.6 and 59.9 h after SC and PO administration, respectively, which implies a longer availability of the drug in lung tissue after SC dosing.

In contrast with florfenicol therapy by continuous drinking water medication during 5 days (Marien et al., 2006; Watteyn et al., 2013b), no re-emergence of the bacterium could be determined in the period after drug administration. These findings can be supported by the PK/PD profile of GAM in turkeys (Watteyn et al., 2015). The results of this earlier study indicated that lung concentrations were above the MIC of GAM against *O. rhinotracheale* strain LMG 9086^T during a rather long period (5 and 10 days after PO and SC administration, respectively). Also after this period, GAM can still be efficacious as it is known that macrolides exert an important post antibiotic effect (Andes et al., 2004). However, *O. rhinotracheale* was detected for a longer period in the tracheal swabs in the treated groups, compared to the CONTR+ group. This might be due to a higher distribution of GAM to lung tissue compared with trachea. However, no PK study of GAM in trachea tissue has been performed.

Recent MIC determinations for GAM revealed that 14 of the 38 *O. rhinotracheale* isolates tested, did not belong to the wild type population, with MIC_{50} and MIC_{90} values of 2 and >32 µg/mL. Consequently, if GAM is used in the field for treatment of *O. rhinotracheale* infections, preferably antimicrobial susceptibility testing should be performed on isolates associated with the disease outbreak. As until now, GAM has not been used in poultry, acquired resistance against this antimicrobial is most probably due to cross-resistance with other antimicrobials. Indeed, depending on the resistance mechanism, cross-resistance may occur with other macrolides, lincosamides and streptogramin B antibiotics (Leclercq and Courvalin, 1991; Zhanel et al., 2001). High MIC_{50} values against *O. rhinotracheale* isolates have been published for the 16-membered macrolides tylosin (32 µg/mL) and tilmicosin (>128 µg/mL), which have been registered for use in poultry in several countries (Devriese et al., 2001; Schwarz et al., 2012).

Conclusion

Although no significant difference of clinical improvement could be noticed, GAM was able to reduce the titres in tracheal swabs in the early stage and in lung tissue compared to the CONTR+ group, suggesting a moderate efficacy of GAM against *O. rhinotracheale* infections in turkeys. This study indicated that a PO bolus administration of the commercially available

product was not as efficacious as a SC bolus. This difference between both routes of administration is likely due to different PK properties of GAM, especially with respect to lung tissue. Therefore, in order to use GAM for oral flock treatment in the poultry industry, dose titration and confirmation experiments and an adapted commercial PO formulation are needed.

Acknowledgments

The authors wish to thank Carine Boone for her technical assistance, and Gunther Antonissen, Nathan Broekaert, Thomas De Mil, Sophie Fraeyman, Elke Gasthuys and Julie Muyle for their aid in the animal experiment.

GENERAL DISCUSSION

To be or not to be, that is the question

Hamlet – W. Shakespeare

Respiratory diseases are a major problem in intensively reared poultry. It is known that these diseases can have multifactorial causes, such as viral and bacterial infections and environmental factors. To control bacterial infections, antimicrobial drugs are often applied in poultry industry. Also for *Ornithobacterium rhinotracheale* infections in turkeys, which can occur from the age of 2 weeks till slaughter, antimicrobials are often used. The frequently used antimicrobial drugs against this pathogen are β-lactams (ampicillin, amoxicillin), macrolides (tylosin, tilmicosin, tylvalosin), tetracyclines (oxytetracyclin, doxycycline) and fluoroquinolones (enrofloxacin). Resistance against the former antimicrobials has been described to some extent. However, florfenicol (FF) and gamithromycin (GAM) are two antimicrobials that have the potential to be effective against *O. rhinotracheale* and which are only registered for use in veterinary medicine, although for the moment only for use in mammals.

The general aim of this doctoral thesis was to evaluate the pharmacokinetics (PK) of FF and GAM, their pharmacodynamics (PD) and their clinical efficacy in an *O. rhinotracheale* infection model. Figure 1 gives an overview of the study designs used, and the main results achieved in this doctoral thesis are summarized in Figure 2.

In this General Discussion, reflections concerning appropriate antimicrobial use and their efficacy are mentioned. In particular, the necessity to use antimicrobials, the route of administration of a drug to poultry and the usefulness of PK/PD models in turkeys are discussed. Afterwards, a general conclusion is made and future perspectives are provided.

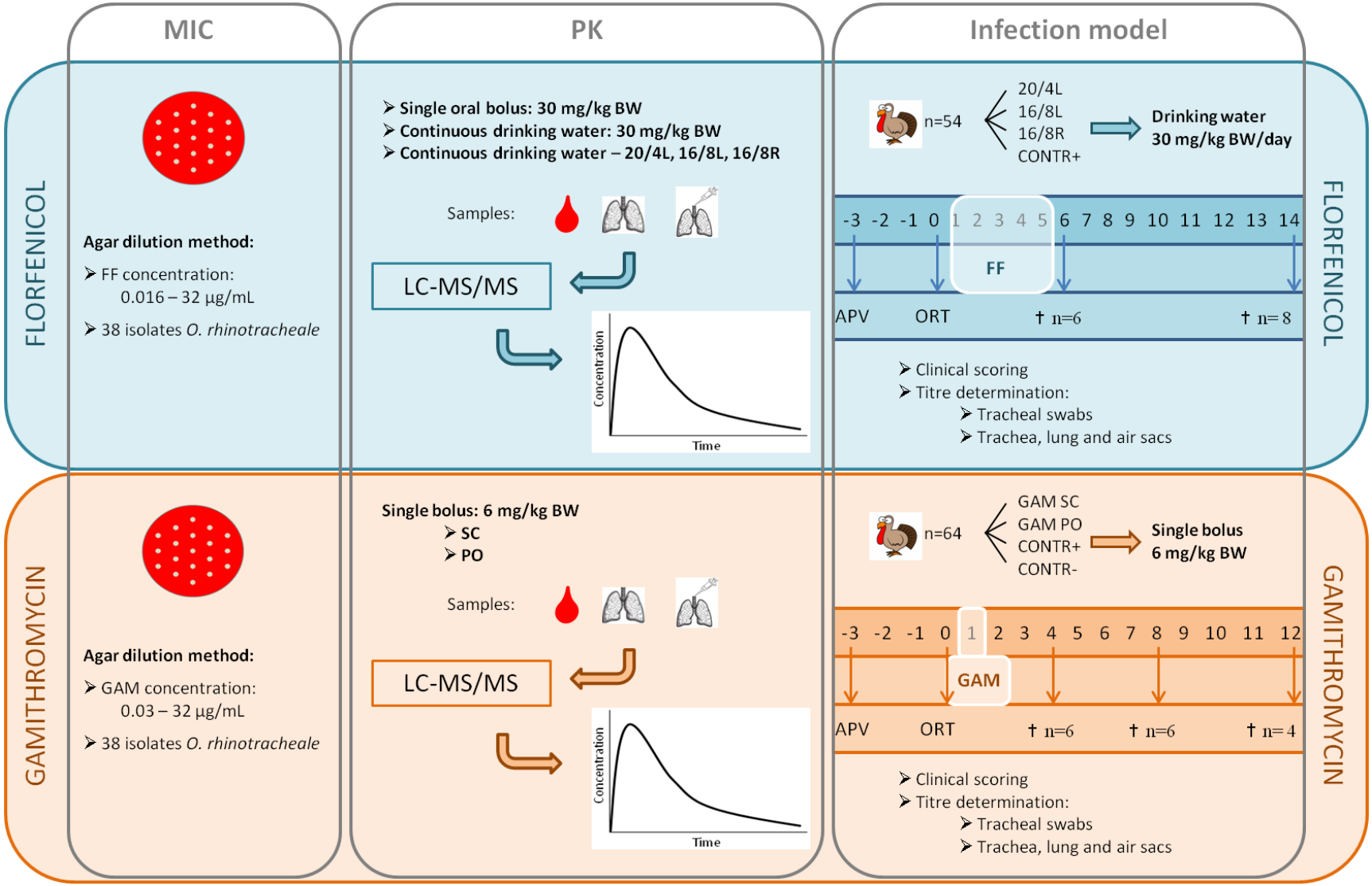

Figure 1. Overview of the study designs used in the present doctoral thesis.

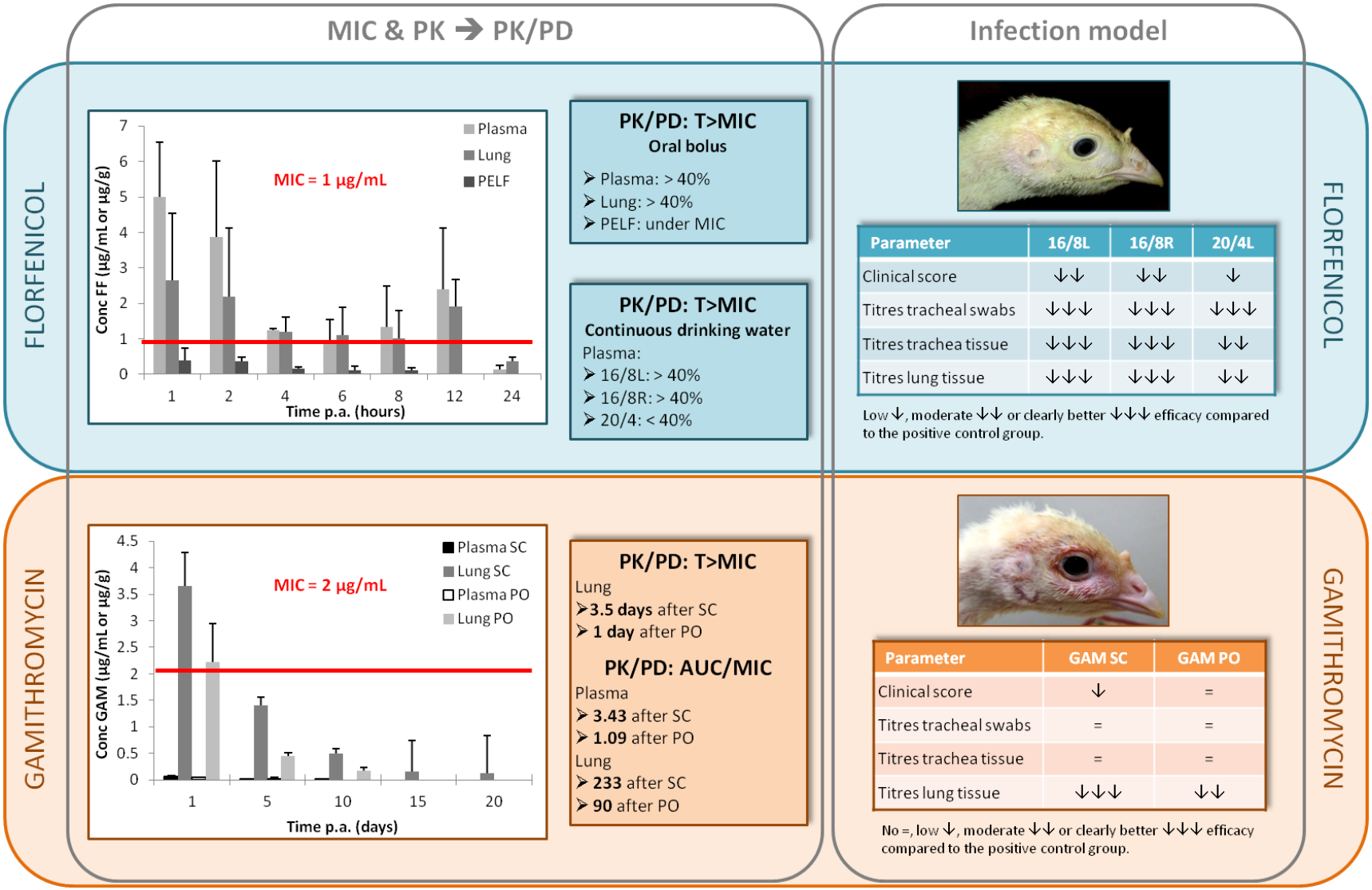

Figure 2. Overview of the main results achieved in this doctoral thesis.

To treat or not to treat

At the end of the latest century, a new concept about comparative medicine was introduced, “One Health”. This can be defined as an approach to improve health and well-being through the prevention of risks and the mitigation of effects of crises that originate at the interface between humans, animals and their various environments (EFSA, 2015). Antimicrobial resistance is one of the topics within the field of One Health, as it is a worldwide concern for both human and animal health (WHO, 2014).

To minimize the resistance selection against antimicrobial drugs, the key question in veterinary medicine is whether it is always necessary to treat with an antimicrobial or whether can the problems be prevented by alternatives such as an improved management. Moreover, if a treatment is necessary, which antimicrobial is the best choice and when does the treatment have to be started?

The impact of **management** practices on poultry health is particularly high, because of the high stocking density during a production round. Optimal housing temperature and humidity as well as low dust and ammonia concentrations are crucial as preventive measures to avoid stress and sickness within the flock (Koknaroglu and Akunal, 2013). In addition, the **photoperiod**, or day/night rhythm, is of great importance for the health status. Longer periods of darkness give a low metabolic rate during this period, resulting in reduced metabolic diseases and consequently lower mortality (Classen et al., 2004). Chickens show a better humoral and cellular immune response in a day and night pattern compared to constant light regimes (Kirby and Froman, 1991; Moore and Siopes, 2000). Also Schwean-Lardner et al. (2013) concluded that lighting programs have an effect on infectious diseases, with a higher morbidity and mortality with increased photoperiod. Hence, an optimal light scheme can result in a reduction of the application of antimicrobials.

To reduce the use of antimicrobials, **probiotics** can be applied to maintain a good health. According to the World Health Organisation (WHO), probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (WHO, 2002). In broiler nutrition, they have a beneficial effect on the digestive tract such as modulation of the intestinal microbiota, pathogen inhibition and intestinal histological changes (Kabir,

2009). They might also exert immunomodulatory properties, with an effect on the humoral and cellular immune response (Koenen et al., 2004). In this respect, there is an indication of potential efficacy on infections beyond the gastrointestinal tract. Oral intake of probiotics can indeed affect the severity of respiratory tract infections in humans, such as a common cold, with a reduction of the duration of symptoms, total symptoms scores and days with fever (de Vrese et al., 2005; Winkler et al., 2005). It can be interesting to study the effect of probiotics on respiratory infections in poultry too. In contrast, **prebiotics** are selectively fermented dietary ingredients, such as non-starch oligosaccharides, that result in specific changes in the composition and/or activity of the gastrointestinal microbiota (Ducatelle et al., 2015). Due to the microbial shift, also the bacterial metabolites can be modified. These metabolites can be absorbed into the blood and enter the systemic circulation, where they can interact with physiological processes of organs and peripheral tissues (Lenoir-Wijnkoop et al., 2007). Whether these prebiotics also have a positive effect on respiratory diseases is still unknown.

In spite of preventive measures such as improved management and the use of pro- and prebiotics, an antimicrobial treatment is often required. Therefore, it is of great importance to make a well-considered selection of the **antimicrobial class**. The World Organisation for Animal Health (OIE) divided all antimicrobials used in food-producing animals in three classes, namely critically important, highly important and important (OIE, 2007). This classification is based on two criteria. The first is a response on a questionnaire whether the antimicrobial class is important. The second criterion is met when compounds within a class are identified as essential against specific infections and there is a lack of sufficient therapeutic alternatives. As the two antimicrobial drugs used in this research, FF and GAM, fulfil criteria 1 as well as 2, they are both classified as critically important antimicrobials.

The phenicols are more used in animals compared to humans (1.05 and 0.087 mg/kg of estimated biomass, respectively) (EFSA, 2015). This is a result of the restricted use of chloramphenicol (CAP) in humans due to severe adverse effects such as irreversible aplastic anemia and bone marrow suppression (Schwarz et al., 2004) and FF is not registered for human use. As CAP is prohibited for use in food-producing animals, FF is the most used subclass in veterinary medicine. The resistance in poultry against FF is rather limited. According to a Belgian report, *Escherichia coli* has a high susceptibility for FF (DGZ, 2015).

Other gastrointestinal bacterial isolates from poultry, such as bacterial pathogens obtained from *Campylobacter* spp. and *Clostridium perfringens*, seemed to be highly susceptible to FF as well (Gholamiandehkordi et al., 2009; Agunos et al., 2013; Kashoma et al., 2014). FF has also a good activity against respiratory pathogens, such as *Pasteurella multocida* and *Mycoplasma gallisepticum* (Aye et al., 2001; Sellyei et al., 2009; Gharaibeh and Al-Rashdan, 2011). The current research showed MIC values of 1 µg/mL against *O. rhinotracheale*. Moreover, the clinical study confirmed the efficacy of FF. Accordingly, these interesting MIC values as well as the successful *in vivo* efficacy study proved that FF would be a good therapy to treat turkeys suffering from an *O. rhinotracheale* infection.

Although macrolides are the second highest selling antimicrobial class in human medicine, the total consumption is even higher in animals (11.51 and 8.72 mg/kg of estimated biomass, for animals and humans respectively) (EFSA, 2015). In contrast to phenicols, the macrolides have several members divided in subclasses, registered for both human and veterinary medicine. This wide application makes the macrolides more vulnerable for resistance selection because of the existence of cross-resistance between macrolides, lincosamides and streptogramin B. The zoonotic pathogen *Campylobacter* can be resistant to veterinary macrolides, such as tylosin, tilmicosin and tulathromycin, but also to the macrolides mainly used in human medicine, such as erythromycin, azithromycin and clarithromycin (Belanger and Shryock, 2007). This high resistance again several macrolides can explain the bimodal MIC distribution of *O. rhinotrachele* for GAM in the current study. Although GAM has never been used in poultry, more than 35% of the tested isolates showed acquired resistance (MIC >32 µg/mL). Also against other macrolides, such as erythromycin, tylosin and tilmicosin, high MIC values in *O. rhinotracheale* has been reported (Devriese et al., 2001; van Veen et al., 2001). An important issue to mention, is that the susceptibility is geographically dependent. Consequently, susceptibility testing of the *O. rhinotracheale* isolate is recommended before starting a treatment. Although the MIC of the used strain in our *in vivo* infection study was low (0.5 µg/mL), the results were not as promising as in the FF experiment. The area under the curve of the clinical scores did not differ significantly from the control group. Also the bacterial titre in trachea did not reduce significantly compared to the control group.

To choose the most appropriate antimicrobial drug, the **mechanism of action** is another important determinant. The activity of antimicrobials can be either bacteriostatic or

bactericidal, but some compounds can exhibit both kinds of effects. GAM administered at normal doses acts bacteriostatic, whereas in higher doses the activity becomes bactericidal. When to administer a bacteriostatic versus a bactericidal antimicrobial depends on the immune response of the host, the bioburden in the host and the pathology of the disease process (Martinez et al., 2013). A bacteriostatic antimicrobial will only be effective in the presence of an adequate host defence. In case of immunosuppression, a bactericidal drug or bacteriostatic drug at bactericidal (higher) dose is recommended (Nemeth et al., 2015). In chickens and turkeys, the immunity can be impaired by environmental factors, such as high ammonia levels or mycotoxins, and infectious diseases, such as infectious bursal disease and Marek's disease (Hoerr, 2010). Therefore, it is of great importance to know the health status of the flock in order to establish a correct treatment. When the bioburden is extremely high and when the bacteria synthesize toxins, the administration of a bactericidal antimicrobial can give rise to even more damage when the high concentrations of toxins are released from the bacterium (Martinez et al., 2013).

An important issue in the treatment strategy is an **optimal dose** for the desired clinical effect. For antimicrobials it is of great importance to increase the efficacy and to reduce the selection pressure for resistance (McKellar et al., 2004). Therefore, dosage strategies have been developed to determine the best dosing regimen (involving dose rate, inter-dosing interval, duration of treatment and modalities of administration) (McKellar et al., 2004). These studies have to be perfomed in the target species as the dosage regimen for a drug in a given species may depend on its anatomy, biochemistry, physiology, and behaviour, as well as on the nature and causes of the condition requiring treatment (Toutain et al., 2010). An efficacious dose (ED) is calculated by PK and PD components, namely the clearance (Cl) and the bioavailability (F) of the drug (both PK parameters) and the efficacious plasma concentration (EC, PD parameter), $ED=\frac{Cl \times EC}{F}$ (Toutain et al., 2010). To determine the best plasma concentration, Monte Carlo simulations could be used with several PK and PD observations.

Beside their anti-infective effects, some antimicrobial agents have also **anti-inflammatory** and **immunomodulatory** effects. These properties could act synergistically with the antimicrobial activity. It has been shown that several macrolides have these properties (Kanoh and Rubin, 2010). Macrolides have been described as potent inhibitors of inflammation induced by lipopolysaccharide (LPS, a structure of the cellwall of Gram-negative bacteria). This action relies on the inhibition of the acute phase response by decreasing the release of cytokines and acute phase proteins (Kanoh and Rubin, 2010). Also pro-apoptotic activities of macrolides were reported (Chin et al., 1998; Lee et al., 2004; Fisher et al., 2011). However, it must be mentioned that this inhibitory effect is mainly studied *in vitro*, and especially *in vivo* studies in poultry are lacking. This has also been reviewed by Plessers et al. (2015b) and Wyns et al. (2015a).

However, for GAM no anti-inflammatory nor immunomodulatory properties could be demonstrated in an intravenous LPS challenge model in pigs and calves (Plessers et al., 2015a; Wyns et al., 2015b). In these studies the animals were clinically scored and also several pro-inflammatory mediators, such as cytokines (tumor necrosis factor α, interleukin 1β and IL-6), acute phase proteins (C reactive protein, haptoglobin, serum amyloid A) and eicosanoides (prostaglandin E2), were determined. For FF, in contrast, Zhang et al. (2008) found that the drug significantly inhibited murine cytokine production both *in vitro* and *in vivo* after LPS challenge.

It would be interesting to study pro-inflammatory cytokines and acute phase proteins to investigate the immunomodulatory properties of these antimicrobial drugs in bacterial infection models, and to find out if these properties are beneficial to cure poultry from a respiratory infection. However, this is not straightforward in poultry. For instance, commercially available ELISA kits for avian cytokines or acute phase proteins are scarce compared to mammalian ELISA kits.

The **start of treatment** could also be of importance as it can influence the exposure to the antimicrobial agent and also the selection of resistance (Ferran et al., 2009). In poultry medicine, a methaphylactic treatment is currently used, which means that all animals in the flock that are exposed to the pathogen, even if some do not display symptoms, are treated. Because of the high stocking density in poultry rearing, metaphylactic treatment of the flock can prevent further spread of the pathogen and cure subclinically infected animals (Hofacre,

2002). This also implies that the bacterial load at that moment targeted by the antimicrobial is lower compared to a later curative treatment, in which all animals are sick. Consequently, lower antimicrobial dosages could be applied to eradicate the bacterial population (Morrissey and George, 1999; Udekwu et al., 2009; Ferran et al., 2011). Moreover, due to the lower prevalence of mutants in these low inoculums, this early start of treatment may lead to a less frequent selection of resistant bacteria (Ferran et al., 2009; 2011).

To drink or not to drink

Drinking water medication is commonly used in the poultry industry to treat sick birds. Moreover, other routes of administration are rarely applied in poultry medicine. Some difficulties are emerging in the manufactering process starting from an active pharmaceutical ingredient towards an efficacious drug, especially for drinking water medication.

Flock treatment, mostly conducted by drinking water medication, is easy to perform, it has a low economic cost and the welfare of the birds is better as they have less stress (Vermeulen et al., 2002). A huge advantage of drinking water medication is the sufficient water uptake when birds are sick, while the feed intake is decreased. However, an individual registration of the intake of water, and the related drug uptake, is not possible under practical conditions. However, Monte Carlo simulations could be used to include the variability in PK and PD of the population. Following, different dosage regimens can be evaluated, resulting in the prediction of an appropriate regimen which will result in the clinical cure of >90% of the animals (Toutain, 2015).

As drinking and eating behaviour depend on the sleeping/waking rhythm, the intake of drugs varies along the day. Consequently, the duration of the photoperiod has an effect on PK properties of drugs administered through the drinking water and feed. Accordingly, also the efficacy of drugs is influenced by the lighting regime. For antimicrobial drugs which are time-dependent, it is important to have plasma concentrations or concentrations at the biophase above the MIC for a sufficiently long period. Concentrations below the MIC can give rise to treatment failure or may induce resistance selection. Thus, in the case of continuous drinking water medication, the photoperiod is of great importance (Santos et al., 1997; Lilia et al., 2008). Indeed, in the research presented in Chapter 1.1, low FF plasma concentrations were observed during the dark period. However, a too short dark period (4 h) can give rise to even lower concentrations. Since 2007, the European Union has layed down rules for the rearing of meat producing poultry. Concerning the photoperiod, the lighting must follow a 24-hour rhythm and should include periods of darkness lasting at least 6 h in total, with at least one uninterrupted period of darkness of at least 4 h, excluding dimming periods (Anonymous, 2007). This doctoral thesis demonstrates that the period of darkness of 8 h is

superior to 4 h of darkness. Considering these findings, it is important for every farmer to know the water intake of their flocks, as these can depend of the environmental conditions (not only of the photoperiod, but also of the temperature or the humidity). Further studies could be conducted to relate other evironnmental factors to the efficacy of antimicrobials.

In case of **individual treatment**, oral or parenteral, only the sick animals receive the drug and this in a correct dosage. However, this is very time consuming and gives more stress to the animals due to the manipulation (Vermeulen et al., 2002). Only in eggs and one-day-old chicks, individual administration is performed in practice (Hofacre, 2002). In addition, it is important for food-producing animals to avoid both tissue damage and the presence of local residues after a parenteral administration, especially for long-acting formulations, such as GAM (Toutain et al., 2010).

In the development of an adequate drinking water medication, the **formulation** of the drug is the most critical part. Ideally, the optimal drug is highly water soluble, homogenous and stable after dissolution, has a high oral bioavailability, a neutral flavour and is palatable. To achieve this goal, the addition of excipientia and/or specific formulation techniques are needed.

For drinking water medication a high water solubility of the active substance could be an advantage, but this physicochemical characteristic often results in low intestinal permeability and oral bioavailability due to the high polarity and poor lipophilicity of those drugs (Martinez and Amidon, 2002). On the other hand, highly lipophilic compounds will typically have a low aqueous solubility, whereas the permeability will be high. To achieve a high oral bioavailability, a balance between these two properties is essential. Several pharmaceutical technical methods can increase the aqueous solubility, such as the formulation of solid dispersions, particle size reduction, complexation, use of hydrophilic carriers or preparations of the amorphous form (Aucamp et al., 2015). In our experiments with FF, the medicated drinking water was prepared by stirring an aqueous solution of FF standard for 30 minutes, followed by sonication for 20 minutes to dissolve the FF. However, these procedures are not feasible in practice. Hence, the major challenge is to develop a water soluble formulation for the lipophilic molecule FF that can be used in poultry practice.

The oral bioavailability of a drug is affected by the rate and extent of its absorption into the enterocytes from the gut lumen, but also by the possible presystemic elimination by the intestine and liver before it reaches the systemic circulation (Kwon, 2001). A low oral bioavailability can be due to gastric instability, low solubility, limited permeability or high intestinal and hepatic first-pass metabolism (Padovan et al., 2012). Also the presence of feed additives, for instance mycotoxin binders, can reduce the oral bioavailability by forming complexes between the drug and the binder (Devreese et al., 2012; De Mil et al., 2015). However, the feeding status of the animal (fed or fasted) and the inclusion rate of the binder can influence the extent of interaction. Although macrolides have excellent PK properties, such as a rapid absorption, very high tissue distribution with high tissue to plasma ratios and a long elimination half-life, the oral bioavailability can be considered low in most animal species. In chickens, tylosin has an oral bioavailability lower than 30% (Kowalski et al., 2002; Ji et al., 2014). The current formulation of GAM, intended for SC use, has also a low oral bioavailability in turkeys. Compared to a SC administration, the relative oral bioavailability was only 25%. As the absolute bioavailability in chickens is 100% after SC use (Watteyn et al., 2013a), we can suggest that this is also 25% after oral administration. If GAM would be considered to be used in poultry medicine to treat respiratory infections, a new and better water soluble formulation has to be developed.

Guggenbichler et al. (1985) stated that the intestinal commensal microbiota is less exposed to the antimicrobial when antimicrobials are administered parentally compared to orally, leading to more limited resistance selection. This is however highly dependend on the PK characteristics of the compound, e.g. oral bioavailability, intestinal secretion and route of excretion (renal *vs* biliair). However, a study with enrofloxacin in broiler chickens demonstrated that the intestinal microbiota is exposed to high levels of the antimicrobial, after oral as well as parenteral therapy (Devreese et al., 2014) although enrofloxacin is mainly renally excreted. Further investigation about the impact of the intestinal levels on resistance selection in the intestinal microbiota is needed.

Whether to treat with a **bolus, single or multiple**, or **continuous administration** of an antimicrobial depends on the activity of the drug.

For a concentration-dependent antimicrobial, for example fluoroquinolones, a high C_{max} and a low t_{max} is advised for a maximal antibacterial effect. To obtain such high plasma

concentrations, a concentrated bolus administration is recommended (Sumano et al., 2003). This could be obtained *via* the normal drinking water system, with the use of a dispenser. To ensure rapid consumption of the medicated drinking water, it is advised to restrict drinking water 1 h before onset.

FF and GAM are both time-dependent antimicrobial agents, in which an extended residence time above the MIC is important to achieve a good efficacy. Hence, continuous drinking water medication is an excellent method to administer FF. In our study, the plasma concentrations in turkeys were below or just around the MIC of 1 µg/mL during continuous drinking water medication at a dose of 30 mg/kg BW/day. Remarkably, after the oral bolus administration of the same dose, the T>MIC was more than 12 h. This suggest that a bolus is a more efficacious method to administer FF, as the disadvantage of a variable uptake is minimized. In practice, a pulsed dosage of 30 mg/kg BW each day, during 5 successive days can be administered in the drinking water to turkeys.

In contrast to FF, GAM is a long acting antimicrobial in which a single bolus could be sufficient to reach adequate concentrations. After single SC administration of 6 mg/kg BW, the plasma levels remained below the MIC of 2 µg/mL. However, the lung concentrations were for a long time (3.5 days) above the MIC. As GAM concentration was not determined in trachea tissue, it is unknown to what extent GAM distributes to trachea tissue. After SC as well as PO administration, GAM was able to reduce the titre of *O. rhinotracheale* in trachea and lung tissue. But only the SC injection could significantly decrease the bacterial titre in lung tissue.

Whether a single oral bolus of GAM would be sufficient to cure from a respiratory infection is still unknown. An appropriate formulation of GAM for oral dosing should be developed and applicated to a higher dose.

To extrapolate or not to extrapolate

PK and PK/PD studies in mammals are more frequently reported compared to poultry, but the extrapolation of the PK properties from mammals to poultry is not straightforward. Some antimicrobial agents are widely distributed to tissues, resulting in higher tissue concentrations compared to plasma. Currently, the PK/PD indices are only based on plasma concentrations, and tissue values are not available. Another variable is the matrix in which the MIC is determined. The value of this *in vitro* PD parameter can vary among determination in broth medium, plasma or even tissue.

Allometric scaling is the study of size and all its consequences which has been used for several decades in the field of drug development (Mahmood, 2005). Laboratory animals, such as mice, rabbits and pigs, are used for selection and screening of pharmaceutical compounds for human medicine. In veterinary medicine, the off-label use of veterinary drugs and eventually human drugs is quite common. This inaccuracy of extrapolation can lead to ineffectiveness or even toxic reactions. Mammals and birds differ in many aspects, such as cardiovascular, hepatic and the gastrointestinal, urinary and respiratory tract. These differences have consequences for the ADME processes of drugs (Dorrestein, 1992; Baert and De Backer, 2003; De Backer, 2006; Neirinckx et al., 2010, 2011). Therefore, **PK studies** in the species of interest are essential to predict the efficacy of drugs.

The anatomy and physiology of the gastrointestinal tract are key determinants for the oral absorption of drugs. In contrast with mammals, avian species have a crop, a glandular stomach and a gizzard. In chickens, the pH in these regions is around 4.5 (Jimenez-Moreno et al., 2009), whereas the pH in the stomach is much lower in mammal species. These differences have an impact on drug absorption based on the ionization of the compound, which depends on the pK_a of the drug (Rivière, 2011). The presence of *Lactobacillus* spp. in the crop can inactivate macrolides, which can result in a lower bioavalability of the drug (Devriese and Dutta, 1984).

The distribution of drugs is affected by plasma and tissue protein binding. Between mammalian and avian species, but also among avian species, there is variety in the occurrence of binding proteins as well as the extent of protein binding (Baert and De Backer, 2003; Antonissen et al., 2015).

Biotransformation can be performed through various metabolic pathways, which can be divided in phase I and phase II reactions. The cytochrome P450 enzyme complex (CYP450) superfamily holds the most important enzymes of phase I biotransformation. In humans, CYP3A is predominant and is responsible for the biotransformation of 50% of the therapeutic drugs (Zhang et al., 1999). The avian isoform, CYP3A37, is 60% homologous to the human CYP3A4 (Ourlin et al., 2000). As a large variability between these CYP enzymes among animal species exists, extrapolation is difficult (Nebbia et al., 2001). Glucuronidation, sulfatation, methylation, glutathione conjugation and amino acid conjugation are major phase II reactions in the biotransformation of drugs. The most important amino acids participating in conjugation reactions are glycine, glutamine, taurine and ornithine (Kasuya et al., 1999). However, ornithine conjugation occurs only in birds and reptilian species, but has not been reported in mammalian species (Igarashi et al., 1992). Even among bird species differences in phase II reactions exist (Baert and De Backer, 2003, Baert et al., 2004). In anseriformes and galliformes the ornithine conjugation is of more importance compared to the glucoronide pathway, whereas in columbiformes the ornithine conjugation is absent (De Backer, 2006).

The total body clearance measures the total ability of the living organism to eliminate a drug. To compare the elimination of drugs between animal species, the relative organ size and relative blood flow of the eliminating organs must be taken into consideration. In general, they both decrease when animal size increases. Since the liver and kidneys are the most important organs for the elimination of drugs, the relative amount of hepatic enzymes and relative number of nephrons/g weight of kidney tissue, which can be defined as the intrinsic clearance, as well as the hepatic and renal blood flow are of great importance. They are all negatively correlated with the body size. Therefore, it is stated that smaller species will eliminate drugs more rapidly compared to large animals (Lin, 1995; Baert and De Backer, 2003; Toutain and Bousquet-Mélou, 2004; Neirinckx et al., 2011; Singh et al., 2011; Antonissen et al., 2015).

Remarkably, it has to be mentioned that the structure of avian nephrons is heterogeneous. At the surface of the kidney, small nephrons with simple glomeruli are located, the so-called reptilian type (RT) nephrons. Nephron size increases with depth from the kidney surface onwards and result in more complex nephrons, the mammalian-type (MT) (Goldstein and Skadhauge, 2000). The sum of the single nephron glomerular filtration rates (SNGFR) is the

whole kidney glomerular filtration rate (GFR). Since the SNGFR increases with the complexity of the nephron, it is higer in MT nephrons compared to RT nephrons. The GFR varies with the body weight (BW) of the animal and for birds following equation can be used for allometric scaling, $GFR = 1.24 \times BW^{0.69}$. Additionally, for the extent of Cl, one can make a distinction between the feeding behaviour of animals, with increasing clearances from carnivores towards omnivores and herbivores (Toutain et al., 2010). In turkeys, the Cl of both FF and GAM was higher compared to mammals. This was also seen in PK studies of other drugs in birds in comparison with mammals, which is consistent with higher metabolic rates in birds (Baert and De Backer, 2003; Toutain and Bousquet-Mélou, 2004; Neirinckx et al., 2011; Singh et al., 2011; Watteyn et al., 2013a).

Beside the specific types of nephrons, avian species also has a specific anatomical characteristic in the kidneys, i.e. a renal portal system. The renal portal vein functions like an artery by carrying blood to the tubules and this is controlled by valves. If drugs are injected into the legs of birds (IM, SC or IV), they can be excreted directly by the renal tubules before entering the systemic circulation (Lumeij, 1994).

The current **PK/PD indices**, T>MIC, AUC/MIC and C_{max}/MIC, have some limitations.

As macrolide concentrations in tissue are often substantially higher compared to plasma concentrations and the PK/PD indices are limited to plasma concentrations, the interpretation of PK/PD analyses is ambiguous. Therefore, PK/PD data at the site of action, are more clinically relevant (Andes et al., 2004; Barbour et al., 2010; Giguère and Tessman, 2011). Still, the interpretation of these results is difficult, as no PK/PD cut-off values for tissues have been established. GAM showed very high AUC/MIC values in lung tissue (233 after SC administration), whereas the plasma values were much lower (only 3.43 after SC administration). Also for other macrolides, low plasma AUC/MIC ratios were correlated with good efficacy (Craig, 2001; Lodise et al., 2005; Martinez et al., 2013). More research should be performed to figure out if the current cut-off values for plasma can be extrapolated to tissue. An antimicrobial could be efficacious in more than one tissue, but to which extent a drug distributes to a specific tissue is not known. Therefore, specific tissue PK/PD could be needed. In contrast, Toutain et al. (2015) argued that plasma or serum concentrations can explain the efficacy of a drug without postulating the tissue as a reservoir. They

demonstrated the applicability of the PK/PD cut-offs of tulathromycin in serum as these cut-off were consistent with the current breakpoints issued by CLSI.

It should be mentioned that the current PK/PD indices T>MIC and AUC/MIC, are obtained for a single 24 h observation time point in steady-state conditions. Some antimicrobial agents, like GAM, act longer than 24 h, implying that this approach is not suitable. Toutain et al. (2007) stated that by dividing the AUC/MIC by the time interval of interest, a more universal metric is obtained. By using this alternative definition, the same metric can be applied to nearly any dosing regimen that may be used in steady-state conditions or single bolus administration. When implementing this information on our data, the distinction in PK/PD index reduces between SC and PO administration, from 233 and 90 to 0.49 and 0.38 for AUC/MIC and AUC/MIC/time, respectively (with time = 480 h and 240 h for SC and PO administration, respectively). Therefore, the value of this index has to be further defined.
Also the use of Monte Carlo simulations to determine PK/PD cut-offs should be more encouraged. This approach will give hypothetical population information and are crucial to come to an optimal dosage regimen.

Nowadays, it is standard practice to determine the MIC in artificial broth, in which there is no influence of *in vivo* matrices, such as binding proteins and pH. However, only the unbound, free fraction of a drug is active. Consequently, when a drug is highly bound to plasma proteins (e.g. tetracyclines), the MIC in plasma could be higher compared to broth, with a lower killing and post antibiotic effect. On the contrary, the MIC of macrolides can be lower in medium supplemented with serum. Bruyck et al. (2012) found that the permeability of *P. aeruginosa* was decreased and the expression of efflux pumps was increased in artificial medium. This results in more resistance against antimicrobials. Also GAM has a better effect in serum, suggesting the existence of a potentiating factor in serum (Mitchell et al., 2013).
The comparison between an artificial and a more physiological relevant matrix is therefore worth to be carried out to know the clinical relevance of MIC values, especially for the classes of antimicrobials of which differences between plasma and broth have already been reported (Mouton et al., 2007; Dorey et al., 2014).

Also breakpoints for veterinary antimicrobials have to be further determined. CLSI has already reported some breakpoints for veterinary pathogens (CLSI, 2013), but it is necessary to extend this list. Also the veterinary division of EUCAST (VetCAST) intends to list breakpoints for antimicrobials for veterinary use in Europe (Veldman, 2015).

The difference between drug analysis in lung tissue versus PELF has been discussed in Chapter 1.2. For antimicrobials which reach high intracellular concentrations, such as macrolides, PELF samples would be better because tissue homogenates could overestimate extracellular concentrations in relation to PELF (Giguère and Tessman, 2011). Although high concentrations of GAM were detected in PELF of cattle as well as foals (Giguère et al., 2011; Berghaus et al., 2011), no GAM could be detected in PELF of turkeys. An explanation could be the different collection method for PELF and the difference in anatomy and physiology of the respiratory tract between mammals and birds. However, the same methodology was used for PELF sampling in the FF study, in which FF was detectable in PELF although at low concentration. Also differences in intracellular distribution of drugs between mammals and birds could explain the very low concentrations of GAM in PELF, as avian macrophages are not constitutively present in PELF (Härtle and Kaspers, 2014). Accordingly, as GAM is more concentrated intracellularly than FF, the GAM concentrations in PELF might be lower.

To conclude and to look forward

According to the presented PK/PD characteristics and in-depth clinical study, it is clear that the use of FF in drinking water can lead to a clinical efficacy for *O. rhinotracheale* infections in turkeys.

For GAM, the PK/PD characteristics seem to be beneficial for the treatment of an *O. rhinotracheale* infection in turkeys, especially for lung tissue, in case of SC administration.

It is generally accepted that drinking water medication is the best option to treat intensively reared poultry. In the current experiments, it was demonstrated that the light scheme is a major issue in the administration of medicated drinking water and drug uptake, which can lead to substantial variability in drug concentrations and efficacy related to different photoperiods.

As the water solubility of FF is low, it is clear that the active substance FF as such is not applicable for drinking water medication. Therefore, it is advisable to develop a water soluble and stable formulation.

Additional research on GAM should also be considered to develop a suitable formulation for oral dosing and to determine an efficient oral dose for GAM in poultry. Moreover, efficacy against other important avian pathogens of the respiratory tract, such as *Mycoplasma* spp, has to be studied as well *in vitro* (MIC determination) and *in vivo* field studies.

It has to be emphasized that PK and PD characteristics, and the related PK/PD indices, of pharmaceuticals have to be evaluated in the species of interest as extrapolation is complex and mostly impossible. Moreover, it is advisable that adjustments for the PK/PD indices for drugs with a high tissue affinity and a long action is required. Also the determination of clinical breakpoints for veterinary pathogens for FF and GAM are requisite for further PK and PD research, as well as the definition of turkey specific PK/PD breakpoint values. Also more elaborate clinical trials with inclusion of placebo controls can give information on clinical breakpoint to define the relationship between measured MIC and probability of cure.

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SUMMARY

Ornithobacterium rhinotracheale is a common avian respiratory pathogen that often affects turkeys during the production round, resulting in significant economic losses. In the course of outbreaks, antimicrobial therapy is applied, but careful attention should be paid to posology, clinical efficacy of antimicrobials and potential antimicrobial resistance.

Florfenicol (FF) and gamithromycin (GAM) are antimicrobial agents which are commonly used in veterinary medicine to treat respiratory infections. Due to their remarkable pharmacokinetic and pharmacodynamic (PK/PD) properties in several mammal species, these drugs may have the potency to cure turkeys from an *O. rhinotracheale* infection.

In poultry, medication is mainly administered through the drinking water. However, when administrating medicated drinking water, drug intake can substantially vary among animals due to animal specific as well as environmental factors, such as light and feeding schemes. Hence, gaining insight into the importance of these factors is mandatory for the establishment of an efficient treatment protocol.

Therefore, the **GENERAL AIM** of this doctoral thesis was to evaluate the pharmacokinetics of FF and GAM, which are both not currently used in poultry, and to study their efficacy against an *O. rhinotracheale* infection in turkeys.

The **GENERAL INTRODUCTION** gives an overview of the respiratory infections in poultry, with a focus on *O. rhinotracheale* infections in turkeys. Currently, the main strategies to control *O. rhinotracheale* infections are the adjustment of management practices and, in case of bacterial outbreak, antimicrobial treatment. Although a vaccine is available, it is not frequently used in the field, due to the seroselectivity of the vaccine. Next, more information about drug administration in poultry in general is given and more specific, the importance of medicated drinking water is emphasised. Subsequently, general principles of PK/PD of antimicrobials are outlined and the physicochemical properties, mechanism of action and resistance, and PK and PD characteristics of FF and GAM are presented.

Since a proper therapeutic effect relies on the understanding of the PK as well as the PD characteristics, it is important to obtain these data in the species of interest. **CHAPTER 1** describes the PK and PD characteristics of FF (**CHAPTER 1.1**) and GAM (**CHAPTER 1.2**) in plasma, lung tissue and pulmonary epithelial lining fluid (PELF) in turkeys, with respect to *O. rhinotracheale*.

After a single oral bolus administration of 30 mg/kg body weight (BW), FF was rapidly absorbed in plasma and rapidly distributed into the respiratory tract (t_{max} = 1.0 h for plasma and lung tissue). The mean half-life of elimination ($t_{1/2el}$) in plasma and lung tissue was similar, around 6 h, whereas it was slightly higher in PELF, namely 8.7 h. In plasma, the mean maximum concentration (C_{max}) was twice as high as in lung tissue, 4.26 µg/mL and 2.64 µg/g, respectively, while in PELF it was much lower, namely 0.39 µg/mL. On the other hand, during drinking water medication (5 days at a dose of 30 mg/kg BW/day), FF concentrations were higher in lung compared to plasma, resulting in lung/plasma concentration ratios above 1. The minimum inhibitory concentration (MIC) of FF for *O. rhinotracheale* was determined at 1 µg/mL for both the MIC_{50} and MIC_{90}. After an oral bolus, the time above the MIC_{90} (T>MIC) was 67.4% and 50.0% of a 24 h interval in plasma and lung tissue, respectively, but PELF concentrations never exceeded the MIC_{90}. After drug administration through drinking water, lung and PELF concentrations were never above the MIC_{90}. Furthermore, during medicated drinking water treatment, different light and feeding schemes were evaluated and an important influence was demonstrated of the photoperiod on the PK of FF. During 16 h of light, the T>MIC was more than 40%, whereas it was less than 40% during a lighting period of 20 h. Therefore, it can be advised that a photoperiod of 16 h is preferred above 20 h to have sufficient FF intake via drinking water. No influence of feed restriction was observed.

The second antimicrobial, GAM, was given as a single subcutaneous (SC) or oral (PO) bolus at a dose of 6 mg/kg BW. The mean plasma C_{max} after PO administration was a ten-fold lower than after SC injection (0.087 and 0.89 µg/mL, respectively). Mean maximum lung concentrations were significantly higher compared to plasma levels for both routes of administration (2.22 and 3.66 µg/g for PO and SC, respectively). Consequently, lung/plasma ratios were up to 50 and 80 after PO and SC administration of GAM, respectively. Although, GAM could not be detected in PELF. The MIC_{50} and MIC_{90} for *O. rhinotracheale* against GAM was determined at 2 and >32 µg/mL, respectively. For PK/PD correlations the MIC_{90} of the susceptible population (2 µg/mL) was taken into account. For lung tissue the T>MIC was 1 day after PO bolus and 3.5 days after SC administration. The area under the curve (AUC)/MIC ratio for lung tissue was 90 and 233 after PO and SC administration, respectively, whereas this was only 1.1 and 3.4 in plasma after PO and SC administration.

In **CHAPTER 2** the efficacy of FF (**CHAPTER 2.1**) and GAM (**CHAPTER 2.2**) was determined in an *in vivo O. rhinotrachelae* infection model in turkeys. In both experiments, the birds were infected with avian metapneumovirus (APV) and *O. rhinotracheale*, followed by a treatment with either FF or GAM.

FF was given as a 5-day treatment at a dose of 30 mg/kg BW/day, administered *via* drinking water. Different photoperiods and feeding schemes were taken into account. During treatment, a clinical improvement could be noticed, resulting in a reduction of the clinical score. The results demonstrated an important influence of the photoperiod on the clinical outcome in an infection model, with significant lower clinical scores in the groups with 16 h of light as compared to the group with 20 h of light. No effect of feed restriction was noticed with respect to the clinical outcome.

GAM was given as a single bolus, either SC or PO, at a dose of 6 mg/kg BW. After SC administration of GAM, a slightly better clinical outcome could be noticed and turkeys recovered earlier compared to those treated with PO GAM and the non-treated group. After SC as well as PO administration of GAM, the bacterial titre of *O. rhinotracheale* in trachea and lung tissue could be reduced. Although, only the SC injection could significantly decrease the bacterial titre in lung tissue.

In **CONCLUSION**, the presented PK/PD characteristics and the results of the clinical studies using a standardised *O. rhinotracheale* infection model, show that FF and GAM can potentially be used in a treatment of *O. rhinotrachelae* infections in turkeys.

Because of the poor water solubility, FF as such is not applicable for medicated drinking water treatment. Therefore, an appropriate pharmaceutical formulation with a higher water solubility and dissolution rate would be preferable. The present studies also confirm the importance of the light scheme in medicated drinking water treatment. With respect to the clinical trials, FF was able to inhibit *O. rhinotracheale* in the trachea and lung tissue, resulting in clinical improvement.

For GAM, the PK/PD characteristics were appropriate to cure turkeys from an *O. rhinotracheale* infection, especially for lung tissue. Indeed, the outcome of the clinical study could confirm the efficacy of GAM against *O. rhinotracheale*, but only in lung tissue and after SC administration.

SAMENVATTING

Ornithobacterium rhinotracheale is een frequent voorkomend respiratoir pathogeen bij pluimvee. Voornamelijk kalkoenen worden hiermee vaak besmet tijdens de opfokperiode, hetgeen resulteert in grote economische verliezen. Bij een uitbraak wordt er een antimicrobiële therapie aangewend, maar hierbij moet steeds aandacht worden besteed aan belangrijke aspecten zoals dosering, klinische werkzaamheid van het antibioticum en mogelijke resistentieselectie.

Florfenicol (FF) en gamithromycine (GAM) zijn antimicrobiële geneesmiddelen die voor de behandeling van respiratoire infecties frequent gebruikt worden in de diergeneeskunde. Door de gunstige farmacokinetische (PK) en farmacodynamische (PD) eigenschappen van beide geneesmiddelen in diverse zoogdieren, zouden deze antibiotica ook gebruikt kunnen worden bij de behandeling van *O. rhinotracheale* infecties bij de kalkoen.

In de pluimveesector dient men geneesmiddelen meestal toe via het drinkwater. Indien gemedicineerd drinkwater wordt aangewend, kan de opname van de actieve substantie echter zeer sterk variëren tussen de dieren onderling, afhankelijk van zowel fysiologische en pathologische omstandigheden bij het dier zelf, alsook omgevingsfactoren, zoals licht- en voederschema's. Daardoor is het nuttig om de invloed van deze factoren te onderzoeken om zo een efficiënt behandelingsschema te kunnen bepalen.

De **ALGEMENE DOELSTELLING** van dit doctoraat was de evaluatie van de farmacokinetische eigenschappen van FF en GAM, die beide momenteel nog geen toepassing kennen in de pluimveesector, alsook het nagaan van hun werkzaamheid bij een *O. rhinotracheale* infectie in kalkoenen.

In de **ALGEMENE INLEIDING** wordt een overzicht gegeven van respiratoire infecties bij pluimvee, met onder meer het aspect van *O. rhinotracheale* infecties bij kalkoenen. Op dit ogenblik zijn de belangrijkste methoden om een *O. rhinotracheale* infectie onder controle te houden in de eerste plaats de aanpassingen van het bedrijfsmanagement en in geval van een bacteriële uitbraak, het gebruik van antimicrobiële middelen. Hoewel een vaccin beschikbaar is, wordt dit in de praktijk niet frequent gebruikt wegens de serospecificiteit van het vaccin. Vervolgens wordt er aandacht besteed aan geneesmiddelengebruik bij pluimvee in het algemeen, en in het bijzonder wordt de toediening via gemedicineerd drinkwater besproken. Nadien worden de algemene principes van PK/PD uitgelegd om tenslotte de fysicochemische

eigenschappen, het werkings- en resistentiemechanisme, en de specifieke PK en PD eigenschappen van FF en GAM te vermelden.

Het is bekend dat de therapeutische efficiëntie van een geneesmiddel zowel afhankelijk is van de PK als van de PD eigenschappen. Daarbij is het evenwel noodzakelijk dat deze data worden verkregen in iedere diersoort waarvoor het geneesmiddel is bestemd. **HOOFDSTUK 1** beschrijft de PK en PD eigenschappen van FF (**HOOFDSTUK 1.1**) en GAM (**HOOFDSTUK 1.2**) in plasma, longweefsel en pulmonale epitheliale vloeistof (PELF) in kalkoenen, met betrekking tot *O. rhinotracheale*.

Na toediening van een eenmalige orale bolus van 30 mg/kg lichaamsgewicht (LG) werd FF snel geabsorbeerd in het plasma en vervolgens snel verdeeld naar de longen (t_{max} = 1.0 u voor zowel plasma als longweefsel). De gemiddelde eliminatie halfwaardetijd ($t_{1/2el}$) in plasma en longweefsel was nagenoeg identiek, ongeveer 6 u, maar was iets hoger in PELF, namelijk 8.7 u. De gemiddelde maximale plasmaconcentratie (C_{max}) was dubbel zo hoog als de C_{max} in longweefsel, 4.26 µg/ml en 2.64 µg/g respectievelijk, terwijl in PELF een veel lagere concentratie werd opgetekend (0.39 µg/ml). In tegenstelling tot de rechtstreekse orale bolus, werden tijdens de drinkwatermedicatie (5 dagen, dosis van 30 mg/kg LG/dag) hogere FF longconcentraties waargenomen dan in het plasma, en was de long/plasma ratio dus hoger dan 1. De minimum inhiberende concentratie (MIC) van FF tegenover *O. rhinotracheale* werd vastgelegd op 1 µg/ml, en dit zowel voor MIC_{50} als MIC_{90}. Na toediening van een orale bolus bedroeg de tijd dat de FF concentratie hoger was dan de MIC (T>MIC) 67.4% voor plasma en 50.0% voor longweefsel tijdens een 24 u interval, maar de PELF concentraties bedroegen nooit meer dan de MIC_{90}. Na toediening van het antibioticum via drinkwater werden zowel in longweefsel als in PELF geen concentraties bekomen boven de MIC_{90}. Tijdens de experimenten met gemedicineerd drinkwater werden ook diverse licht- en voederschema's bestudeerd. De studies toonden aan dat het lichtschema een belangrijke invloed had op de PK van FF. De T>MIC bedroeg meer dan 40% wanneer 16 u licht werd voorzien, terwijl bij 20 u licht dit lager bleek dan 40%. Daaruit kan worden geconcludeerd dat een periode van 16 u licht te verkiezen is boven een 20 u lichtcyclus om tot een optimale opname van FF via drinkwatermedicatie te komen. Invloed van voederschema's op de PK van FF werd niet waargenomen.

Het tweede antibioticum, GAM, werd toegediend via een eenmalige subcutane (SC) of orale (PO) bolus aan een dosis van 6 mg/kg LG. Na toediening van de orale bolus was de gemiddelde C_{max} in plasma tien maal lager dan na SC injectie (0.087 en 0.89 µg/ml, respectievelijk). De gemiddelde maximale longconcentraties waren significant hoger in vergelijking met de plasmaconcentraties voor beide toedieningswijzen (2.22 en 3.66 µg/g na PO en SC, respectievelijk). Zo waren long/plasma ratio's tot 50 en 80 na respectievelijk PO en SC toediening van het antibioticum. GAM kon echter niet gedetecteerd worden in PELF. De MIC_{50} en MIC_{90} van *O. rhinotracheale* voor GAM werden vastgelegd op 2 en >32 µg/ml, respectievelijk. Voor de PK/PD correlatie werd de MIC_{90} van de gevoelige populatie (2 µg/ml) in beschouwing genomen. Zo bleek de T>MIC in longweefsel 1 dag na PO en 3.5 dagen na SC toediening te bedragen. De oppervlakte onder de curve (AUC)/MIC ratio voor longweefsel was 90 en 233 na respectievelijk PO en SC toediening, terwijl voor plasma slechts waarden van 1.1 en 3.4 werden opgetekend na respectievelijk PO en SC toediening.

In **HOOFDSTUK 2** werd de werkzaamheid van FF (**HOOFDSTUK 2.1**) en GAM (**HOOFDSTUK 2.2**) nagegaan in een *in vivo O. rhinotracheale* infectiemodel in kalkoenen. In beide experimenten werden de dieren geïnfecteerd met aviair metapneumovirus en *O. rhinotracheale*, en werden ze vervolgens behandeld met ofwel FF of GAM.

FF werd continu toegediend gedurende 5 dagen aan kalkoenen via drinkwatermedicatie aan een dosis van 30 mg/kg LG/dag. Verschillende licht- en voederschema's werden toegepast. Gedurende de behandeling werd een duidelijk klinische verbetering waargenomen. De resultaten toonden ook een belangrijke invloed van het lichtschema aan op de klinische score, met significant betere klinische resultaten in de groepen met 16 u licht ten opzichte van de groep met 20 u licht. Een effect van de verschillende voederschema's werd niet vastgelegd.

GAM werd toegediend als een eenmalige SC of PO bolus, aan een dosis van 6 mg/kg LG. Na de SC bolus, kon een lichte klinische verbetering waargenomen worden en de dieren vertoonden hierbij ook een sneller herstel in vergelijking met de PO en niet-behandelde groepen. Door toediening van GAM kon, na zowel SC als PO toediening, de bacteriële titer van *O. rhinotracheale* in trachea en longweefsel worden verminderd. Evenwel, enkel na SC injectie van GAM werd de bacteriële titer in longweefsel significant verlaagd.

Als **CONCLUSIE** kunnen we stellen dat de verkregen PK/PD eigenschappen en de resultaten van de klinische studies, uitgevoerd in een gestandaardiseerd *O. rhinotracheale* infectiemodel, het mogelijke gebruik van FF en GAM staven bij *O. rhinotracheale* infectie bij kalkoenen.
De wateroplosbaarheid van FF is echter vrij laag, waardoor dit geneesmiddel als dusdanig momenteel niet toepasbaar is voor drinkwatermedicatie. De ontwikkeling van een geschikte farmaceutische formulatie met een hogere wateroplosbaarheid en oplossnelheid dient zich dan ook aan. Bovendien werd in deze studies ook een belangrijke invloed van de lichtcyclus op de toediening van drinkwatermedicatie bevestigd. In de klinische studies kon door toediening van FF *O. rhinotracheale* duidelijk geïnhibeerd worden ter hoogte van zowel trachea als longweefsel, met als gevolg gunstige klinische resultaten.
Voor GAM waren de PK/PD eigenschappen gunstig om kalkoenen met een *O. rhinotracheale* infectie te behandelen, zeker ter hoogte van het longweefsel. In de klinische studie kon de werkzaamheid van GAM tegenover *O. rhinotracheale* worden aangetoond, maar uitsluitend ter hoogte van het longweefsel en enkel na SC toediening.

CURRICULUM VITAE

Anneleen Watteyn werd geboren op 27 oktober 1982 te Gent. Na haar middelbare studies, Moderne Talen – Wetenschappen aan het Sint-Hendriks en Zusters Maricolen Instituut te Deinze, startte ze in 2000 met de studie Biomedische Wetenschappen aan de Kulak. In 2004 behaalde ze met onderscheiding het diploma van Master in de Biomedische Wetenschappen aan de KULeuven. Onmiddellijk daarna vatte ze de studie Diergeneeskunde aan aan de Universiteit Gent, waar ze in 2008 met onderscheiding afstudeerde als dierenarts, optie Gezelschapsdieren.

Geboeid door het onderzoek, startte Anneleen in 2009 als assistent aan het laboratorium voor Farmacologie en Toxicologie van de vakgroep Farmacologie, Toxicologie en Biochemie. Haar onderzoek handelde over de farmacokinetische en farmacodynamische eigenschappen van enkele antimicrobiële middelen, met daaraan gekoppeld de studie van de efficaciteit van deze antimicrobiële middelen in een respiratoir infectiemodel bij de kalkoen. Tijdens haar assistentenmandaat stond Anneleen mee in voor de farmacologische en toxicologische dienstverlening van de vakgroep. Daarnaast verzorgde ze ook de praktische oefeningen Farmacokinetiek in 3de Bachelor Diergeneeskunde en was ze promotor van meerdere masterthesissen. In 2015 vervolledigde ze het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Anneleen is auteur en medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en presenteerde haar onderzoeksresultaten op verschillende internationale congressen.

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DANKWOORD

Als je de tocht aanvaardt naar Ithaka
wens dat de weg dan lang mag zijn,
vol wederwaardigheden, vol belevenissen.
De cyclopen en de Laistrygonen,
de woedende Poseidon hoef je niet te vrezen,
zulke ontmoetingen zul je nooit hebben op je weg.
Wanneer je denken verheven blijft,
verfijnt de emotie die je hart en lijf beroert.
De cyclopen en de Laistrygonen,
de woeste Poseidon zul je niet tegenkomen
wanneer je ze niet in je eigen geest meedraagt,
wanneer je geest hun geen gestalte voor je geeft.

Wens dat de weg dan lang mag zijn.
Dat er veel zomermorgens mogen komen
waarop je heel dankbaar, heel blij
onbekende havens zult binnenvaren.
Dat je mag pleisteren in Fenicische handelssteden
om mooie dingen aan te schaffen
van parelmoer, koraal, barnsteen en ebbenhout,
en opwindende geurstoffen van alle soorten,
opwindende geurstoffen zoveel je krijgen kunt.
Dat je talrijke steden in Egypte aan mag doen
om veel, heel veel van de geleerden op te steken.

Blijf wel altijd denken aan Ithaka.
Daar aan te komen is je doel.
Maar overhaast de reis in geen geval.
’t Is beter dat die vele jaren duurt en
je pas als oude man bij het eiland afmeert,
rijk door wat je onderweg verwierf,
zonder verwachting dat Ithaka je rijkdom schenken zal.

Ithaka schonk je de mooie reis.
Bestond het niet, dan was je nooit vertrokken.
Maar meer heeft het je niet te bieden.

En vind je het armzalig, Ithaka bedroog je niet.
Zo wijs geworden, met zo veel ervaring
heb je al wel door waar Ithaka’s voor staan.

Uit Ilias van Homeros

Zoals Homeros beschreef in zijn heldendicht *Ilias*, is de weg naar Ithaka belangrijker en rijker dan de aankomst op het eiland zelf. Zo ook heb ik een fantastische reis meegemaakt de afgelopen jaren. Uiteraard bleef deze dag mijn doel, maar de kennis en vriendschappen die ik opgebouwd heb, zijn veel meer waard.
Gedurende de jaren zijn er heel wat mensen met mij meegereisd, voor even of voor de hele trip, elk met hun eigen invulling. Zo werd deze reis onvergetelijk! Daarvoor wil ik jullie dan ook heel graag bedanken!

Allereerst wil ik mijn promotoren bedanken, zonder hen was er uiteraard geen sprake van een doctoraatsthesis.
Prof. dr. Croubels, Siska, jouw deur staat altijd open, voor een toxicologische vraag, voor een zoveelste versie om na te lezen, of gewoon voor een babbel. Na al die jaren aan de vakgroep heb ik veel van jou geleerd en heb ik je enorm leren waarderen, voor je wetenschappelijke gedrevenheid, je welbekende oog voor detail, maar ook voor je steun en optimisme bij moeilijke momenten. Bedankt om er al die jaren te zijn!
Prof. dr. De Backer, bedankt dat ik aan de vakgroep mocht starten met mijn eigen onderzoek. Het heeft wat voeten in de aarde gehad, maar we zijn er uit geraakt. De vele historische en culinaire weetjes tijdens de vakgroepuitstappen en congressen zullen me altijd bijblijven.
Prof. dr. Devreese, Mathias… wie had er ooit gedacht dat je zo vroeg in mijn dankwoord ging komen. Bedankt om me de laatste jaren bij te staan bij de dierproeven, de analyses en het neerpennen van de resultaten! Je was een enorme meerwaarde als promotor van mijn thesis. Veel succes nog in je verdere carrière en natuurlijk ook thuis, dat alle wegen mogen opengaan ☺.

Ook aan de leden van de examencommissie wil ik mijn oprechte dank betuigen voor het kritisch nalezen van dit werk en hun sterk gewaardeerde input in mijn onderzoek.
Prof. dr. Ducatelle, Rik, de fundamenten voor mijn interesse in diergeneeskunde zijn bij jou en Justine gelegd. Bedankt om als voorzitter alles in goede banen te leiden. **Prof. Dr. Pelligand, Ludo**, thank you for making time to come over from the UK and for your contributions to the PK/PD integration. **Prof. dr. Vanrompay**, bedankt om tijd vrij te maken voor het lezen van mijn thesis en voor de suggesties. **Dr. Boyen, Filip**, bedankt voor je tips &

trics bij de MIC bepalingen. **Dr. Van Hoorebeke, Sebastiaan**, door jou wist ik altijd wel kalkoenen te vinden. Bedankt voor je advies en om de thesis kritisch na te lezen door de ogen van een praktijkdierenarts!

Prof. dr. Meyer, Evelyne, bedankt om mij 7 jaar geleden op te bellen voor de vervanging van Heidi en zo mijn carrière aan de vakgroep te laten starten. Na de ommezwaai van mijn doctoraat, was de link met de biochemie wat minder, maar toch bedankt voor je blijvende interesse in mijn onderzoek.
Prof. dr. Haesebrouck, **Prof. dr. Pasmans** en **Prof. dr. Martel**, bedankt voor de input in het realiseren van de dierproeven, en de waardevolle suggesties bij het nalezen van mijn artikels.

Een grote dank je wel is zeker op zijn plaats voor de doctoraatscollega's. Met hen verliepen de dierproeven vlotter, ging het labowerk wat sneller vooruit, werd het werk eventjes opzij gezet tijdens de lunches en waren er aangename koekjespauzes op donderdagnamiddag.
Allereerst mijn bureaugenootjes Sophie, Elke en Joske, bedankt voor jullie luisterend oor, jullie aanmoedigingen en de aangename vrouwenbabbels. **Sophie**, mijn assistentenmaatje, samen hebben we heel wat cases uitgeklaard en talloze telefoons gepleegd (de een al enthousiaster dan de ander ☺). Bedankt voor de vele fijne jaren, gevuld met chocolade-shots, caffeïnegeur en gerimpelde appeltjes. **Elke G.**, onze google-madam, niets is voor jou te veel! Jij hebt me de laatste jaren met zoveel zaken geholpen, van synoniemen vinden, over varkensaangelegenheden tot persoonlijke chauffeur van deze thesis! Bedankt voor al jouw hulp en je enthousiasme! Aan jou laat ik ook met plezier mijn taak als vakgroepuitstap-planner na ☺. **Joske**, de avonturier die net op tijd terug is ☺. Bedankt voor de gezellige momenten en nog veel plezier in de slachthuizen en met de oh-zo-leuke Potter ☺. Meisjes, draag zorg voor onze kalender en onze plantjes!!!
Gunther, jouw pluimveekennis is niet te evenaren! Bedankt voor je weetjes, de oh-zo-gezellige dierproeven met monsters van beesten ☺ en de plezante congresuitstappen.
Ook bedank ik graag onze buren, de "jongensbureau", kwestie van de gender-neutraliteit op peil te houden op de vakgroep! Bedankt voor jullie vele hulp! **Nathan**, veel plezier op je wereldreis samen met Marlien, helemaal op jouw ritme "alles kan, niets moet" ☺. **Thomas**, veel succes met het afwerken van je doctoraat en natuurlijk ook in je nieuwe job. **Joren**,

sinds kort mijn stadsgenoot, veel succes nog met de varkentjes en de str*-analyses. We komen elkaar wel nog eens tegen in Deinze (in de Colruyt of zo ☺)!
Wim, een stoere collega met een klein hartje ☺, de naalden zullen wel wennen na verloop van tijd! **Marianne**, beetje bij beetje overwon je je angst voor onze gevederde vrienden en voor de kleine roze monstertjes. Bedankt voor je enthousiasme en gulle lach!
Sandra, de laatste aanwinst van onze vakgroep, veel succes met je onderzoek! Als meter maak ik je graag wegwijs op ons labo.

Naast de mededoctoraatsstudenten waren er natuurlijk ook de andere collega's aan ons labo die elk op hun manier bijgedragen hebben tot dit doctoraat.
Een héél grote dank je wel aan **Siegrid**! Zonder jouw analytisch vermogen en werkkracht waren de analyses van de geneesmiddelen niet gelukt. Ook onze sportieve uitjes op donderdagmiddag waren nodig om eventjes stoom (letterlijk en figuurlijk) af te laten. **Jelle**, bedankt om die allerlaatste validaties nog uit te voeren volgens de GLP standaarden (zo was alles heel duidelijk voor mij ☺), maar ook om mij in te wijden in de wondere wereld van de toxicologie. **Julie**, jouw opgewektheid is een plezier om te ervaren in de stal en in het labo. Bedankt voor je hulp! **An M.**, onze labo-perfectioniste, jij leerde mij om alles piekfijn uit te voeren in het labo. Bedankt voor je opmerkingen (die soms wel nodig waren ☺) en je advies, maar evenzeer voor je luisterend oor! **Ann S.**, een warme persoonlijkheid die we nu wat minder zien, maar bedankt voor je gulle lach en de oorbelletjes ☺. **Marc**, bedankt voor de leuke babbels in het labo. **Kris**, dank je wel voor al jouw pluimvee- en kinetiekkennis, en natuurlijk ook voor de overheerlijke honing. **Filip**, ondanks de stijgende stapels aankoopbonnen, facturen, tox-verslagen blijft je enthousiasme enorm groot om steeds klaar te staan als administratieve hulp. Merci daarvoor!

Mijn dank gaat ook uit naar de "overkant van de gang", de collega's van de Biochemie.
Kristel, bedankt om mij "in de tijd" de geheimen van flowcytometrie en ELISA mee te geven, natuurlijk ook voor jouw enorme gezelligheid en no-nonsense praatjes tijdens de lunch en koekjespauze. **Jorien**, bedankt voor jouw heerlijke chocoladecakes en gezellige babbels over zussen en hondjes. Héél veel succes met die soms-vervelende artikels en op naar de laatste spurt! **Koen** en **Jonas**, het muizenduo van de vakgroep. Veel succes nog met jullie baanbrekend kankeronderzoek. En Koen, jouw kennis en doorzettingsvermogen zal mij altijd

bijblijven! **Femke**, helaas geen IWT-beurs, maar hopelijk kan je aan onze vakgroep blijven. Veel succes!

A great appreciation to our Italian friends, Elisa and Matteo. **Elisa**, your work was the start of the ORT-subject. Thank you for your contribution to my PhD during your stay in Belgium! **Matteo**, thank you for your help with the FF-analyses and validation on lung tissue! Molto grazie per tutti!

Gedurende die 7 jaar, zijn er natuurlijk heel wat mensen de revue gepasseerd. De laatste die bij ons wegging was **Nathalie**, een trouwe kracht als het op administratie aankwam. Bedankt voor de vele onkostennota's, facturen en de gezellige babbels in het secretariaat! Hopelijk komen we elkaar nog eens tegen op de sportnamiddag van UGent! **Maggy**, bedankt om mij die eerste jaren altijd een vrolijke morgen te bezorgen en de laatste jaren worden we nog steeds verwend met zoetigheden. **Sandra**, ik heb jouw assistentenplaats, inclusief kippen-onderzoek, overgenomen. Helaas, of gelukkig? ☺, heb ik het LPS-gedeelte achter mij gelaten, toch bedankt voor al jouw kennis. **Virginie**, onze onderzoeken lagen ver uiteen, maar dat deed er niet toe tijdens de lunch/koekjespauzebabbels over de kindjes en onze mannen! Nog eens bedankt voor je DGZ-input omtrent ORT! **Ann O.**, bedankt voor de vele gezellige uren tussen onze gevleugelde vriendjes, het was TOP! Jouw kipje staat nog steeds te blinken op mijn bureau, en het zal ook doorgegeven worden aan de volgende kippen-onderzoeker! **Joline** en **Anja**, de olijke tandem. Ondertussen zijn jullie en onze wegen al wat verder uit elkaar, maar ik hoop dat we op tijd en stond tijd vinden voor een brunch of plons-break. Allebei succes met jullie al-niet-zo-nieuwe job en vele fantastische jaren met jullie mannen aan jullie zijde gewenst! **Veerle** en **Nermin**, een korte stand-in, maar door jullie hulp in het labo, is mijn onderzoek net iets vlotter gegaan, bedankt! En Nermin, probeer je West-Vlaams te onderhouden tussen die Hollanders daar! De ex-collega's van de Biochemie, **Jella**, **Donna**, **Bert** en **Dieter**, bedankt voor de aangename samenwerking. Jella en Donna, wij komen elkaar nog wel eens tegen, in de Resto of tijdens een AAP-vergadering! Succes met jullie onderzoeken!

Het bacteriologische luik van deze thesis was niet tot stand gekomen zonder de hulp van de diensten Bacteriologie en Pluimveeziekten. In het bijzonder **An G.**, bedankt om mij in te wijden in de wereld van de kalkoenen, de trachea-staalnames en om steeds paraat te staan om al mijn vragen te beantwoorden. **Arlette** en **Marleen**, bedankt voor de hulp in het labo met de MIC bepalingen!
Ook **Filip**, **Wolf** en **Celine** verdienen een woordje van dank. Bedankt voor de gezellige uitstapjes in Nantes en Stavanger. Wolf, als je nog eens moeilijk uit je bed geraakt, bel mij gerust ☺.

Dan zijn er nog ex-collega'tjes die net iets meer zijn, vriendinnen of zelf "Zusters van de DDD". **Eva**, jij bent mijn allereerste link met de vakgroep. Als promotor van mijn thesis maakte je mij warm om verder in het onderzoek te gaan. Samen hebben we leuke uurtjes beleefd binnen en buiten de muren van de Hoogbouw. Voor jou begint er nu een nieuw hoofdstuk in jullie nieuwe nest. Maak er samen met Dagmar en Elmer een warme thuis van! **Heidi**, van stille mede-studente, over gezellige zuster, toegewijde mama, ongelooflijk gebeten onderzoekster, tot sterke vrouw! Zo heb ik je leren kennen en enorm appreciëren! Bedankt voor alle hulp in de stallen en in het labo, de nee-niet-ziek-enkel-nachtelijke-bloedpunten momenten en de kind-uitstapjes in het weekend. Succes met je job en je uitgebreide gezinnetje, en binnenkort komen we wel eens naar het zeetje! **Elke P.**, de gedreven wetenschapster voor wie geen enkele tox-vraag te veel was! Bedankt voor je hulp met de kippen en kalkoenen, de keuvelmomentjes tijdens de koekjespauze, de tips & trics bij de finalisatie van dit doctoraat, en vooral voor je warme aanmoedigende woorden de laatste maanden!

Sunny, of is het Prof. dr. Eloot ☺, mijn steun voor de wetenschappelijke publicaties, maar ook voor talloze tips voor ontspannende reisjes (met dé Karl!) en gezellige kaas & wijn-avonden (als het in de agenda staat ☺). Bedankt voor het nalezen! Nu kan je je volledig verdiepen in de PK bij kalkoenen!

Een bijzonder woordje van dank aan **Vicky**. Zonder jou was het hier thuis niet gelukt! Bedankt om al die jaren ons huisje piekfijn te maken!

Daniel en Henriette, de liefste buren die we ons konden wensen! Bedankt om ons uit de nood te helpen met een patatje, om de kindjes af en toe eens op te vangen en om mijn stalbroeken te repareren. Dat we nog lang een babbeltje mogen doen aan “ons hekje”.

En dan de “Moederkes”, **Ciska**, **Marlies**, **Nele C.**, **Caroline**, **Lore**, **Nele D.G.** en **Lieke.** Dat blauwe hemd met oranje sjaal bracht ons op een of andere manier bij elkaar. Bedankt voor de vele ontspannende momenten! De etentjes, work-outs, cocktailparty’s of filmavonden, ze waren allemaal even gezellig en nodig om alles even van mij af te kunnen zetten. Een speciaal woordje van dank aan Nele C., mijn sportbuddy, bedankt om er al zoveel jaren te zijn, op school, in de KSA en tijdens onze babbelmomentjes in de BBB (sorry Cis ☺). Onze uitdaging voor het komende jaar is de 11 km!

Chloë & **Peter**, **Robbe** en **Arthur**, jullie kan ik niet genoeg bedanken! Jullie staan steeds paraat om ons te komen depanneren bij een opvang-probleempje. Bedankt voor de zaterdagochtend afspraakjes, de gezellige avondlijke etentjes, de klinkende glazen op Nieuwjaar, en zo veel meer. Binnenkort gaan we er weer op uit naar de Ardennen!!!

Lies & **Benne**, **Fé** en **Cai**, ook jullie wil ik bedanken voor de afgelopen jaren! Twintig jaar geleden begon onze vriendschap en in al die tijd is er heel wat moois uit voortgevloeid ☺. Bedankt voor al die gezelligheid en ik kijk uit naar de komende feestjes!

Mamie en **Bonpapa**, ook voor jullie een speciaal woordje van dank. Ik denk met veel plezier terug aan onze maandagmiddaglunches (met die blonde god ☺), de verblijfjes aan zee tijdens de blok, de verwennerij met verse soep of snoepjes. Dank je wel voor al jullie steun en we gaan er samen nog een Duvelke en een Ricardke op drinken (of toch een porto ☺)!

De zusjes… **Liesbeth**, wij gaan al het langste mee, van ‘malse billetjes’, over een paar ruzietjes ;-) tot het uitwisselen van baby-ervaringen. Zus, **Nicolas**, **Juna** en **Marieke**, dank je wel voor de steun en de warmte in jullie peperkoekenhuisje!
Charlotte, onze avonturier. Bedankt voor de no-nonsense babbels, het nalezen van mijn artikels en de gezellige onderonsjes bij de kalkoenen. Voor jou is het studentenleven nu ook

afgelopen, en trek je er weer op uit. Volg je hart naar waar dan ook, wij komen zeker langs, er is slechts 8983.89 km tussen Deinze en San José ☺!

Elise, ons jongste zusje. Ook aan jou een dank je wel om mij/ons te steunen de afgelopen jaren (nog eens een dikke merci voor al die waterflessen ☺!) en om te komen babysitten zodat ik toch eventjes mijn energie kwijt kon tijdens het sporten. Nu is het aan jou om een paar jaar (5+…) op die universiteitsbanken te zitten ☺.

Mama en **Papa**, hier sta ik dan, na 6+6+4+4+6 jaar studeren, met de grootste zelfvoldoening ☺! Het lange studeren hebben we alle 4 van niet ver ;-) Dank jullie wel om mij al die kansen te geven en om mij en ons gezinnetje te steunen en bij te staan wanneer we het nodig hadden. Zonder jullie was dit niet gelukt!

En als laatste, mijn 4 musketiers… een stoere prinses, een sloeber-dondergod, een lieve blonde krijger en mijn alles. **Amber**, ik sluit een lange schoolcarrière af, terwijl jij er net aan begonnen bent. Ik weet dat je dat niet goed… maar SUPER goed gaat doen! **Thor** en **Finn**, mijn jongens, jullie kunnen nog even genieten van véél spelen, beetje ruziemaken en de wereld ontdekken per fiets of met die kleine voetjes. Dank jullie wel om met een lach en een traan ons leven kleur te geven.

Stijn, in al die jaren hebben we samen vele watertjes doorzwommen (van het Grevelingenmeer tot de Rode zee ☺), knopen (en bomen ☺) doorgehakt en vele bergen verzet. Na 6 jaar hebben we samen deze top bereikt. Het was niet altijd even gemakkelijk, maar bedankt om in mij te blijven geloven, om van ons huis een thuis te maken en om er gewoon te zijn voor mij! Nu is het aan jou!

Ik zie jullie graag, helemaal tot aan de maan… en terug!