FACULTY OF VETERINARY MEDICINE
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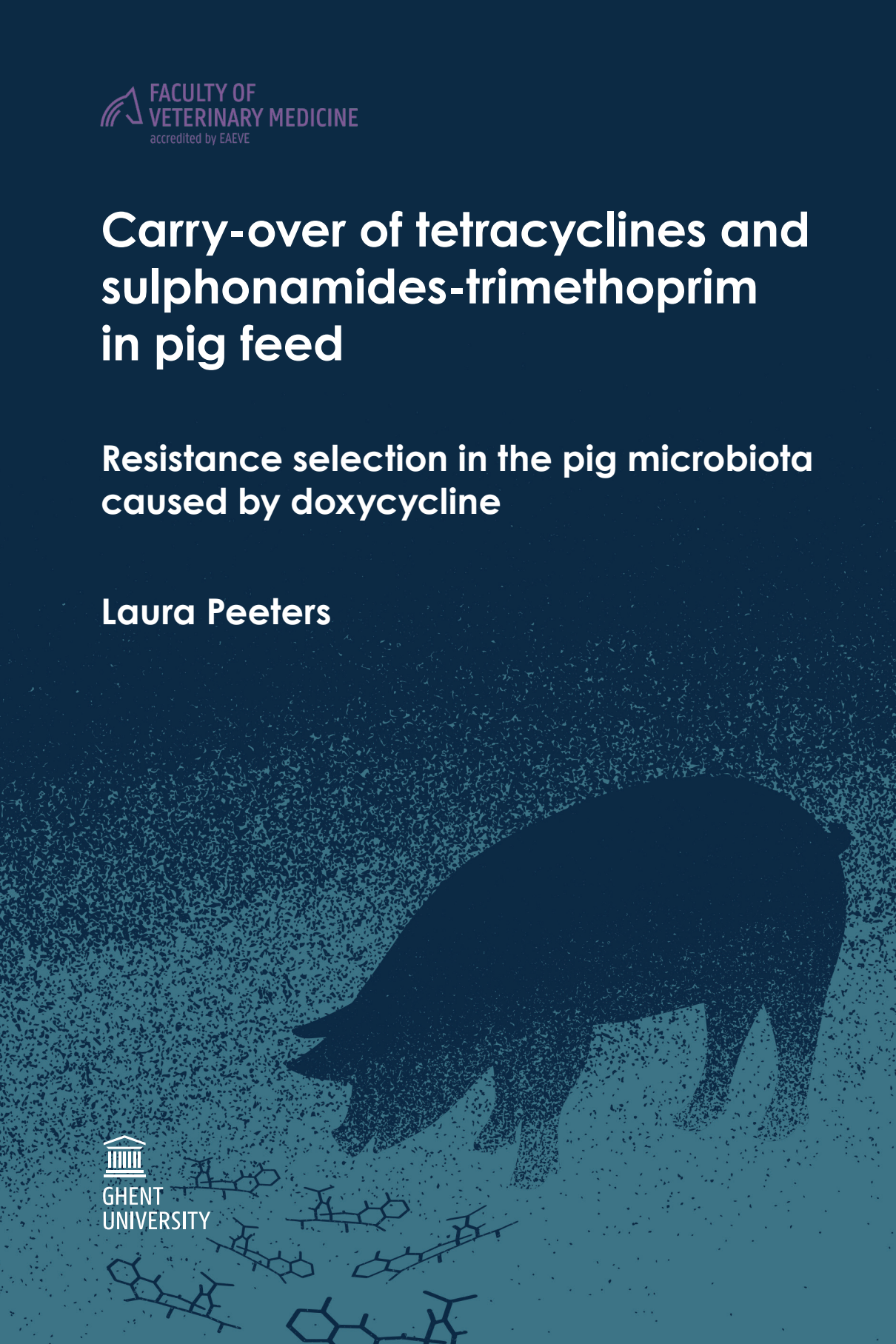
Resistance selection in the pig microbiota caused by doxycycline

Laura Peeters



GHENT UNIVERSITY

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Laura Peeters (2018)

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LIST OF ABBREVIATIONS

A

AGRI	Committee on Agriculture and Rural Development
ALARA	as low as reasonably achievable
AMCRA	Belgian centre for Antimicrobial Consumption and Resistance in Animals
ANOVA	analysis of variance
API	active pharmaceutical ingredient

B

BelVetSAC	Belgian Veterinary Surveillance of Antimicrobial Consumption
BEUC	Bureau Européen des Unions de Consommateurs
BFA	Belgian Feed Association
BW	body weight

C

CCl_3COOH	trichloroacetic acid
CD	colon descendens
CFU	colony forming units
CH_3CN	acetonitrile
CH_3COOH	acetic acid
$C_4H_6O_4$	succinic acid
CH_3OH	methanol
CM	caecum

COGECA	General Committee for Agricultural Cooperation in the European Union
COPA	Committee of Professional Agricultural Organisations
CS	conserved segment
CTC	chlortetracycline

D

DCA	distal colon ascendens
DDDvet	defined daily doses animal
DMCTC	demethylchlortetracycline
DNA	deoxyribonucleic acid
DOX	doxycycline
DOX^R plasmid	doxycycline resistance conferring plasmid

E

EC	European Commission
ECOFF	epidemiological cut-off value
EF	elongation factor
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPIC-PCR	exon-primed intron-crossing polymerase chain reaction
ESI	electrospray ionisation
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EUCAST	The European Committee on Antimicrobial Susceptibility Testing

EURL-AR	EU Reference Laboratory for antimicrobial resistance

F

FAO	Food and Agricultural Organisation of the United Nations
FASFC	Federal Agency for the Safety of the Food Chain
FDA	Food and Drug Administration
FDS	fine dosing system
FEFAC	European Feed Manufacturers' Federation

G

g	goodness-of-fit
GTP	guanosine triphosphate

H

HCOOH	formic acid
HLB	hydrophilic-lipophilic balance
HPLC	high-performance liquid chromatography

I

ICE	integrative and conjugative element
IME	integrative mobilizable elements
IS	internal standard
ISCR	insertion sequence common region

L

LB	Miller's LB broth
LC-MS	liquid chromatography – mass spectrometry
LC-MS/MS	liquid chromatography – tandem mass spectrometry
LOD	limit of detection
log *P*	logarithm of the partition coefficient between octanol and water for the neutral form of the compound
LOQ	limit of quantitation

M

MBC	minimal bactericidal concentration
MC	MacConkey n°3 agar
MF	medicated feed
MIC	minimum inhibitory concentration
MPC	mutant prevention concentration
MRD	maximum recommended dose
MSC	minimal selective concentration
MS/MS	tandem mass spectrometry

N

NADP(H)	nicotinamide adenine dinucleotide phosphate
Na_2SO_4	sodium sulphate
NEVEDI	Nederlandse Vereniging Diervoederindustrie

O

OD_{600}	optical density measured at wavelength 600nm
OIE	World Organisation for Animal Health

P

PABA	para-aminobenzoic acid
PCA	proximal colon ascendens
PCR	polymerase chain reaction
PCU	population correction unit
PD	pharmacodynamic(s)
PK	pharmacokinetic(s)
pKa	acid dissociation constant
PNEC	predicted no effect concentration
PVDF	polyvinylidene fluoride membrane

R

R^2	Pearson's correlation coefficient
^{R}CA	reinforced clostridial agar
Rep-PCR	repetitive extragenic palindromic polymerase chain reaction
RIF	rifampicin
RPP	ribosomal protection protein
R/S	ratio of resistant to susceptible bacteria
RSD	relative standard deviation
RSD_r	repeatability
RSD_R	within laboratory reproducibility

S

SD	standard deviation
SDZ	sulphadiazine

T

Tn	transposon
t_R	room temperature
TR	transfer ratio
TRIM	trimethoprim

U

UPLC	ultra performance liquid chromatography

W

WHO	World Health Organization

CHAPTER 1

General introduction

1 ANTIMICROBIAL USE IN VETERINARY MEDICINE

1.1 INTRODUCTION

The term 'antimicrobial agent' or 'antimicrobial' has been defined by the World Organisation for Animal Health (OIE) as an active substance of synthetic or natural origin which destroys bacteria or suppresses their growth or their ability to reproduce in animals or humans, excluding antivirals and antiparasites (OIE 2011). This is consistent with the wording used by, among others, the Codex Alimentarius (FAO 2011) and the European Food Safety Authority (EFSA) (EFSA 2009). This definition will be applied throughout this thesis.

Antimicrobials are used extensively in both human and veterinary medicine for treatment and prevention of bacterial infections and to improve production efficiency in food-producing animals. The latter is not approved anymore in the EU since 2006 (EP 2003) but is still common practice in countries like the US and China. Antimicrobials are one of the most important tools available to modern medicine. However, soon after the discovery of antimicrobials, it became clear that bacteria could acquire resistance to these products. This phenomenon, antimicrobial resistance, is defined as the ability of a microorganism to multiply or persist in the presence of an increased level of an antimicrobial agent relative to the susceptible counterpart of the same species (FAO 2011). Today, the use of antimicrobials in humans and animals is recognized worldwide as a major driving force in the emergence and spread of antimicrobial resistance. Throughout the years, awareness grew that the selection of a suitable antimicrobial and dosage is a crucial step in any therapeutic regimen, both in animal and human medicine (Collignon *et al.* 2016). To assist veterinarians

in their efforts towards a judicious use of antimicrobials, national and international guidelines on the prudent use of antimicrobial agents have been published (AMCRA 2013, EC 2015).

Although the extent to which different sectors (human, animal, environment) are contributing to the levels of resistant bacteria in other sectors is still unclear, there is a general awareness that immediate action is required to counteract the spread of antimicrobial resistance. This action implies in the first place the reduction of antimicrobial use in all sectors and prevention of the spread of resistance (Collignon *et al.* 2016, Laxminarayan *et al.* 2013). Therefore, international, national and local organizations developed guidelines that request for action (AMCRA 2014, EC 2011, OIE 2016, WHO 2015). Recently, a new Belgian legislation concerning the conditions for the use of veterinary drugs by veterinarians and animal keepers was established (Royal Decree 2016). This new legislation also implies measurements to support the policy on reduction of antimicrobial resistance. The critically important cephalosporins (3rd and 4th generation) and fluoroquinolones can exclusively be prescribed after antimicrobial susceptibility tests have shown that no other antimicrobials would be effective, and, it is forbidden to use these antimicrobials for preventive purposes.

All actions to counter the selection and spread of antimicrobial resistance fit in the more broad principle of the 'One Health principle', a strategic framework that has been established in 2008 to combat the spread of infectious diseases that emerge (or re-emerge) from the interfaces between animals and humans and the ecosystems in which they live. The strategic framework is guided by key principles that include the adoption of a multidisciplinary, multinational and multisector approach; the integration of technical, social, political, policy and regulatory issues; and the establishment of broad-based partnerships across sectors and along the research-to

delivery continuum. Hence, the engagement of wildlife and ecosystems interests, the human and veterinary medical community, as well as advanced research institutions is crucial to make the One Health concept successful (FAO and OIE 2008).

1.2 ANTIMICROBIALS USED IN PIG PRODUCTION

1.2.1 INTRODUCTION

An antimicrobial class is defined as a group of antimicrobial agents with related molecular structures, often with a similar mode of action because of interaction with a similar target and thus subject to similar mechanisms of resistance. The properties of antimicrobials within a class can vary as a result of the presence of different molecular substitutions, which confer various intrinsic activities or various patterns of pharmacokinetic and pharmacodynamic properties (FAO 2011).

Although sulphonamides were the first produced clinically successful broad-spectrum antimicrobials, it was the first large scale production of penicillin in World War II that led to the antimicrobial revolution (Giguère 2013). At the same time, veterinarians started treating bovine mastitis with intramammary infusions of penicillin G, the first of the many representatives of the group of penicillins that were to come. Shortly after the war, streptomycin and chlortetracycline were introduced in food animal production (Gustafson *et al.* 1997). Several tetracycline derivatives became available later on, and they are still used extensively in veterinary medicine, especially doxycycline, a second generation tetracycline (see below, 1.2.2.). The high consumption rate of tetracyclines unavoidably creates a high risk on selection of tetracycline resistant bacteria.

Nowadays, a wide range of antimicrobials is available for use in animal and human medicine. The antimicrobials used to treat or prevent disease

in animals are essentially the same as those in human medicine, although some antimicrobials are reserved for use in human medicine exclusively. The World Health Organisation (WHO) recommends that the use of antimicrobials judged to be essential for human medicine should be restricted and their use in food-producing animals should be justified by culture and susceptibility results (WHO 2000, WHO 2015, WHO 2016). The same accounts for essential antimicrobials for veterinary medicine, e.g. pleuromutilins, which are the only option to treat swine dysentery (*Brachyspira hyodysenteriae*) (OIE 2015).

1.2.2 QUANTITATIVE DATA ON USE OF VETERINARY ANTIMICROBIALS

Concerns about the spread of antimicrobial resistance have led to the **monitoring of trends in antimicrobial consumption** in most high-income and some middle-income countries through databases on **antimicrobial sales**. Sweden (Swedres-Svarm 2017) and Denmark (DANMAP 2017) were the first countries to start collecting veterinary antimicrobial sales data in the 90's, later followed by other European countries, i.e. The Netherlands (MARAN 2017), Norway (NORM-VET 2017) and Belgium (BelVetSAC 2017). Total sales of antimicrobials for food-producing animals have also been reported for non-European countries as the US (FDA 2016), Canada (CIPARS 2016) and Australia (Australian Government 2014). Obtaining reliable data on antimicrobial consumption per animal species based on sales data is complicated because (1) most antimicrobials are approved for use in multiple food-animal species, (2) off-label use (EC 2001) of antimicrobials is a common practice and (3) the sold antimicrobials may not have actually been administered to the animals (Cameron *et al.* 2016). The European Medicines Agency (EMA), that collects national consumption data

from EU-countries in the **European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project**, uses the Population Correction Unit (PCU) to normalize sales data (EMA 2016). The PCU takes into account the size of the animal population and the average theoretical weight of the animal species at the time of treatment. Data on sales of antimicrobial veterinary medicinal products normalised by the PCU are expressed in mg of active ingredient by PCU (mg/PCU) and 1 PCU equals 1 kg of livestock (EMA 2016). However, it is generally accepted that normalisations with the harmonised Defined Daily Doses Animal (DDDvet) as numerator (DDDvet/PCU) are the preferred reference values to compare antimicrobial use between countries and animal species. Efforts have already been made to calculate harmonised DDDvet values on European level for pigs, cattle and poultry. These assigned DDDvet values are often a compromise, as doses can differ between countries, indications, administration routes and formulations.

Figure 1 gives an overview of the total sales of the different antimicrobial classes in the EU (29 countries) in 2014. The largest amount sold was represented by the tetracyclines (33.4%), followed by penicillins (25.5%) and sulphonamides (11.0%). These antimicrobials are considered essential and are listed as veterinary critically important antimicrobial agents by the OIE (OIE 2015). The **pharmaceutical forms** for group treatment accounted for 91.6% of the total sales in the EU; premixes accounted for 42.1%, oral powders for 31.7% and oral solutions for 17.8%. Different formulations for oral treatment have been developed to enable different types of treatment, i.e. individual or group treatment and feed or water medication (see 1.2.4). The availability of formulation also depends on the physicochemical or pharmacokinetic properties of the antimicrobial (see 3). The preferred formulation types for an antimicrobial can vary significantly between countries. This depends largely on the cost-effectiveness, policy and regulations at national level (EC 2016).

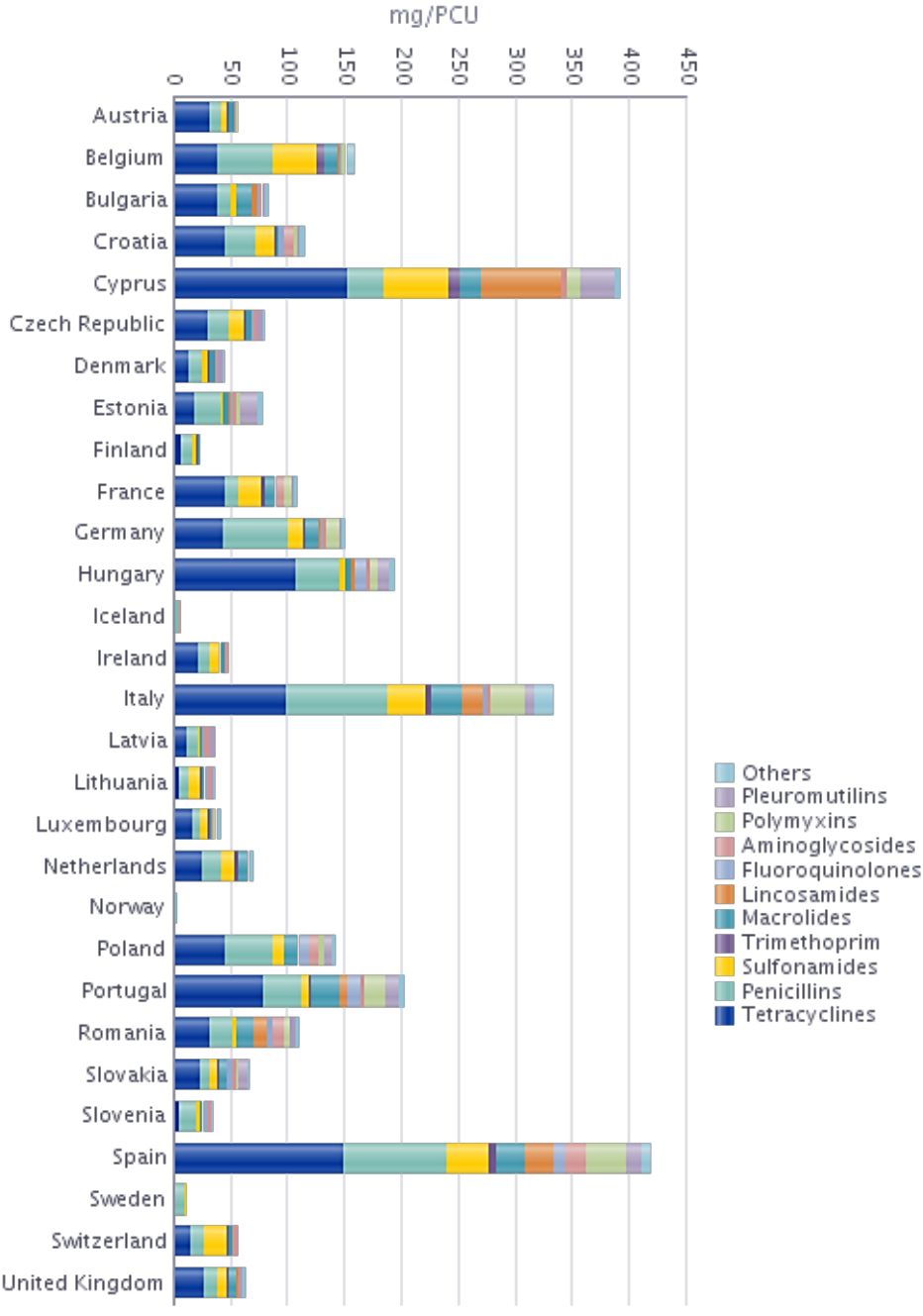


Figure 1. Sales for food-producing species (including horses) in mg/PCU (Population Correction Unit), of the various veterinary antimicrobial classes, by country, for 2014 (EMA 2016).

The distribution of pharmaceutical formulations for tetracyclines and sulphonamides sold in Europe are shown in **Figure 2** and **Figure 3**, respectively. The Belgian Veterinary Surveillance of Antibacterial Consumption (BelVetSAC) showed that sulphonamides, penicillins and tetracyclines have been sold most frequently as premix formulations over the past years, as shown in **Figure 4** (BelVetSAC 2017).

Significant efforts have been made to reduce the use of antimicrobials in livestock and this resulted in an overall fall in sales (mg/PCU) of 12% from 2011 to 2014 (from 138 mg/PCU in 2011 to 121 mg/PCU in 2014) in the participating 24 countries of the EU, excluding Spain. Sales data from Spain between 2011-2013 are thought to be underestimated. Therefore, sales data from Spain are not directly comparable with the other countries and are excluded from the aggregated data. The observed decline was mainly

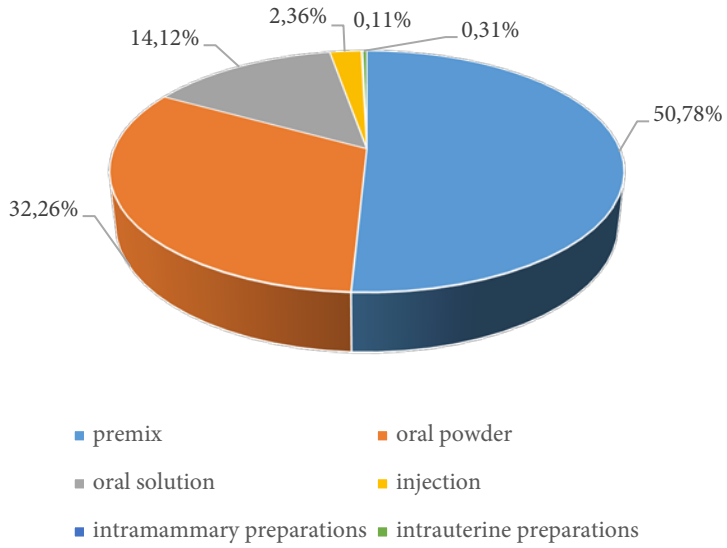


Figure 2. Distribution of sales of tetracyclines for food-producing animals (including horses), in mg/PCU (Population Correction Unit), by the major pharmaceutical forms sold, aggregated by EU/EEA countries for 2014 (EMA 2016).

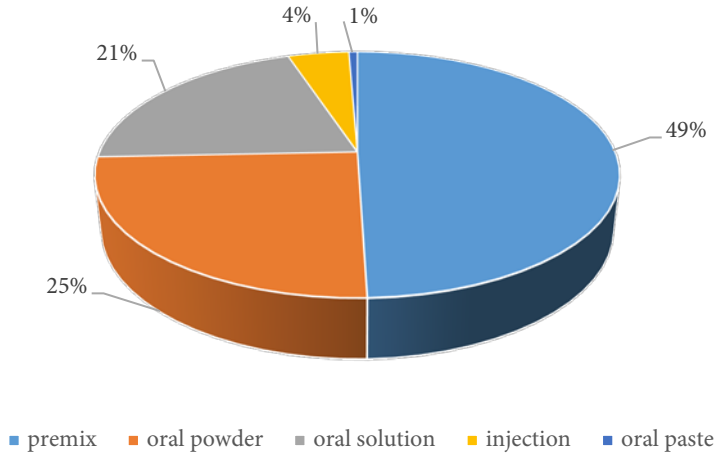


Figure 3. Distribution of sales of sulphonamides for food-producing animals (including horses), in mg/PCU (Population Correction Unit), by the major pharmaceutical forms sold, aggregated by EU/EEA countries for 2014 (EMA 2016).

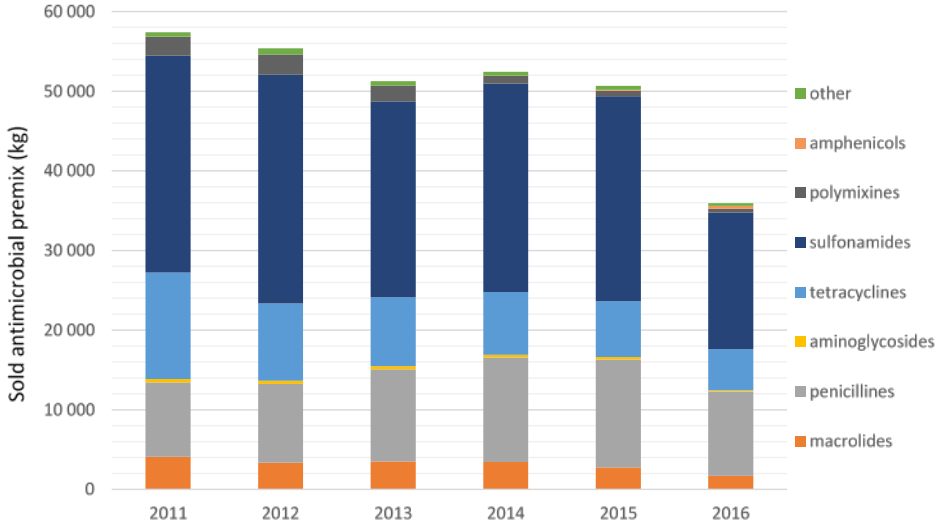


Figure 4. Use of antimicrobials premixes per class of antimicrobials between 2011 and 2016 in Belgium (BelVetSAC 2017).

caused by the implementation of responsible-use campaigns, changes in animal demographics, changes in systems for collecting data, restrictions of use, bench-marking, increased awareness of the threat of antimicrobial resistance, and/or the setting of targets (EMA 2016).

Data on antimicrobial use worldwide are very scarce and certainly incomplete. However, a statistical model estimated that, based on maps of livestock densities and reports of antimicrobials consumption in high-income countries, 63,151 (± 1,560) tons of antimicrobials were globally used in food-producing animals in 2010 (Van Boeckel *et al.* 2015). Per kilogram of produced animal, this represents approximately 45 mg, 148 mg, and 172 mg for cattle, chicken, and pigs, respectively. Global antimicrobial consumption was estimated to rise to up to 150,596 (± 3,605) tons in 2030 (i.e. an increase of 67% compared to 2010), based on growth projections of the consumption of livestock products. This presumed increase would be mainly a result of the growing number of livestock in countries such as Brazil, Russia, India, China and South-Africa (Van Boeckel *et al.* 2015). Antimicrobial use in fish and shrimp farming was not included in this study, although it might be a major source of antimicrobial contamination of the aquatic environment.

More and more countries nowadays make efforts to monitor antimicrobial use on **farm level**. Today, 15 European countries, amongst others Belgium, and Canada are thus collecting data on antimicrobial use per animal species. Fifteen of these countries are registering antimicrobial use in **pig production** in particular (AACTING 2018). In Belgium, a preliminary study on antimicrobial use in pig production has been performed in 2003 (Timmerman *et al.* 2006), which led to recommendations on correct dosing, the expression of concern on the high number of prophylactic group treatments and a proposal to replace these treatments by other disease-preventing measures. However, a study in 2010 on antimicrobial use in

Belgian pig production detected an overall higher use of prophylactic antimicrobial group level therapy compared to 2003 (Callens *et al.* 2012), which indicated that the guidelines for prudent use were not implemented. Moreover, 82% of the administered doses assessed in this study were not according to the label. Starting from 2011, the **Belgian centre for Antimicrobial Consumption and Resistance in Animals (AMCRA)** has set the goal to achieve a general **reduction of antimicrobial use** in animals with 50% by 2020, a reduction of 75% of critically important antimicrobials for human medicine by 2020 and a reduction of 50% of antimicrobial premixes by 2017 (AMCRA 2014). Although the annual BelVetSAC reports on antimicrobial use in Belgium show a clear reduction in antimicrobial use since 2011 (total cumulative reduction of 20.0%), serious efforts will be needed to achieve the goals (BelVetSAC 2017).

1.2.3 PURPOSES OF ANTIMICROBIAL USE IN PIG PRODUCTION

Pig production in the EU is based on intensive production systems in which animals are housed in large groups. The low profit margins drive pig producers to keep their production as efficient as possible. This, together with the constant threat of possible disease outbreaks, has led to a high use of antimicrobial agents in pig production. Moreover, the broad availability of less expensive or cheap generic formulations of antimicrobials of which patents are expired encourages improvident use of these drugs.

The treatment and prevention of bacterial infections by antimicrobials can be achieved in three different ways: therapy, metaphylaxis or prophylaxis (EPRUMA 2013).

In case of **therapeutic use**, the modes of application of the antimicrobial agent depend on the size of the group, animal production type and type of infection. These treatments are often individual, e.g. in sows, although in some cases group treatment of sows can be necessary, e.g. in case

of highly virulent infections (Schwarz *et al.* 2001a). Therapeutic antimicrobial therapy should be preceded by an examination of the diseased animal, preferably accompanied with laboratory analysis such as identification of the pathogen and antimicrobial susceptibility testing. Individual treatment has the advantage of a better control over the appropriate dosage (Page *et al.* 2012, Schwarz *et al.* 2001b). Unfortunately, individual therapy is not a practical option for food-producing animals as they are mostly held in large groups, such as pigs and poultry. In these large groups, an intervention is required as soon as one of the animals presents symptoms of a disease as one can be sure that the others will be affected too (Schwarz *et al.* 2001b). **Metaphylaxis** is defined as the treatment of an entire group of animals after diagnosis of clinical disease in part of this group, with the aim of treating the clinically sick animals and controlling the spread of disease to animals in close contact and at risk which may already be (subclinically) infected (EPRUMA 2013). Ideally, other interventions such as quarantining clinically ill animals should be considered before starting metaphylactic treatment. In contrast to therapeutic and metaphylactic treatment, **prophylaxis** is a solely preventive measure, not preceded by a diagnosis (Page *et al.* 2012, Schwarz *et al.* 2001b). Animals are generally more susceptible to infections at certain stages of life, and especially in the young population (which is the majority of the livestock) when the acquired immunity is not yet fully developed. Some of these crucial periods in pig production are vaccination, weaning, castration and transport and/or mixing of animals (Callens *et al.* 2012, McEwen *et al.* 2002, Schwarz *et al.* 2001a). However, this approach becomes more and more controversial, because antimicrobial (group) treatments exert a high selective pressure on resistant bacteria and their resistance genes (McEwen *et al.* 2002, Schwarz *et al.* 2001a, Schwarz *et al.* 2001b).

1.2.4 ANTIMICROBIAL ADMINISTRATION METHODS IN PIGS

Administration routes

Depending on the purpose of the treatment, antimicrobials can be administered to animals through different routes. They can be administered topically, directly to the site of the infection (EPRUMA 2014), but more common is the systemic use, which implies both oral and parenteral administration. The parenteral route includes intravenous, intramuscular, transdermal and subcutaneous injection (EPRUMA 2014, Page *et al.* 2012). **Table 1** gives an overview of different routes and purposes of antimicrobial administration.

Table 1. Worldwide purposes of antimicrobial treatment and administration routes in swine

Purpose	Individual or group treatment	Administration routes
Prophylaxis	Group	Oral (feed or water medication)
Metaphylaxis	Group or subgroup	Oral (feed or water medication)
Therapy	Individual or subgroup	Injection or oral (feed or water medication)
Growth promotion	Group	Feed medication

Adapted from Page *et al.* (2012).

Group treatment vs. individual treatment

The choice of administration route is usually related to the purpose of the treatment and location of the infection (Schwarz *et al.* 2001b). As mentioned above, prophylactic and metaphylactic treatments are inherently group treatments, whereas therapeutic treatments mostly involve individual treatment of a limited number of animals. Seen the housing conditions

of most food producing animals and the epidemiology of most bacterial infections, it is clear that group treatments are practiced more often.

Parenteral treatment is the preferred administration route to achieve an accurate dosage and treatment duration, as this method does not depend on the eating and drinking behaviour of the animals. Parenteral group treatments are rarely applied, except in case of groups of suckling piglets and calves, as this method is rather labour-intensive (FASFC 2013, Page *et al.* 2012).

For practical reasons, group treatment is generally achieved through oral administration of the antimicrobial via the feed or drinking water (Schwarz *et al.* 2001b). **Feed medication** in particular is a very easy method of group medication from a logistical and labour technical point of view. On the other hand, this method has many disadvantages. Feed intake of animals can be variable, and anorexia in sick animals can result in underdosing. Furthermore, a poor health status can compromise the pharmacokinetic parameters after oral administration, as was shown e.g. for oxytetracycline (Pijpers *et al.* 1991). Other disadvantages are dependent on the type of mixing procedure (FASFC 2013, Page *et al.* 2012). The first procedure, which is the most commonly used procedure in Belgium, is the mixing of the medicated feed in a licensed feed mill on veterinary prescription. Advantages of this procedure are the more accurate dosage of the antimicrobial and the homogeneity of the feed. Nevertheless, this procedure has several disadvantages (FASFC 2013). The risk of segregation of the antimicrobial is rather high because of the many stages of transport the feed must pass before is it consumed. Segregation is a consequence of physicochemical properties such as particle size and electrostatic forces of the different feed compounds including the antimicrobial and medicated premix (FASFC 2013, Page *et al.* 2012). Moreover, there is a risk of carry-over of

antimicrobial residues to non-medicated feed, at the feed mill, during transport and during storage (Filippitzi *et al.* 2016).

Another important disadvantage of mixing at the feed mill is the inflexibility of the therapy. First, the therapy cannot be started before the medicated feed is delivered. Moreover, the farm silo cannot be filled with the medicated feed before the previous feed is entirely consumed (FASFC 2013). Second, the therapy duration depends on the rate of feed consumption, because the complete medicated feed batch needs to be consumed before new (blank) feed can be introduced. Hence, it is often not possible to end the therapy at the proper time. Furthermore, if the feed silo is not entirely empty before the delivery of a new batch of feed, mixing of the two batches of feed can occur, which would lead to incorrect dosage and prolongation of the therapy (FASFC 2013). Third, a large number of animals is usually connected to the same feed line, consequently it is not possible to administer the feed to a subgroup of animals (FASFC 2013).

Finally, the stability of some antimicrobials can be compromised due to the storage of the medicated feed in a silo, e.g. in summer when temperatures in the silo rises. These potential stability problems have not been quantified before, but it has been reported that sulphadimidine and oxytetracycline remain stable in feed in normal conditions, i.e. < 30°C. Moreover, potential interaction of the antimicrobial with feed components may reduce the oral bioavailability of the drug, e.g. chelation of tetracyclines with multivalent ions (FASFC 2013) (del Castillo 2013).

Similar issues arise when the antimicrobial is mixed with the feed at the level of the silo, although this method could reduce the risk of segregation. The use of mobile dosing systems to add the antimicrobial to the feed at the level of the feed line on the farm reduces the risk of segregation even more as the feed does not have to be stored in a silo. Also, this system im-

proves the flexibility of feed medication, with respect to both therapy duration and treatment of specific groups of animals. Unfortunately, this method also has an important disadvantage as the control over dosage and homogeneity is very poor (van Krimpen 2007).

Group medication can also be applied via the **drinking water**. This method requires some more effort from both veterinarian and farmer because several factors need to be taken into account to determine the correct dosage. Average water consumption, the physicochemical properties of the water (hardness, pH), antimicrobial solubility and stability in water, pharmaceutical formulation used, water flow, etc. can influence water uptake and oral bioavailability of the antimicrobial. It is also recommended to measure beforehand the daily water uptake of the group of animals that needs to be treated, because the uptake is variable and depends on various factors such as age and ambient temperature. Moreover, the dosing systems need to be calibrated regularly, and the condition and purity of the water pipes needs to be controlled. Drinking water systems that are not maintained properly can lead to variable antimicrobial doses between different drinking points (FASFC 2013, Page *et al.* 2012, van Krimpen 2007).

A very important advantage of water medication compared to feed medication is the flexibility of the therapy start and duration and the opportunity to select smaller groups of animals for therapy. Moreover, water uptake of sick animals tends to be more stable than feed uptake. Although the absorption of antimicrobials from drinking water is variable and depends on the molecule, it is generally better than the absorption from feed, which is translated in a higher bioavailability (van Krimpen 2007).

2 CROSS-CONTAMINATION OF FEED

2.1 MEDICATED FEED AND CROSS-CONTAMINATION

'Medicated feed' is defined as a mixture of one or more veterinary medicinal products or intermediate products with one or more feeds which is ready to be directly fed to animals without further processing. An intermediate product is defined as a mixture of one or more veterinary medicinal products with one or more feeds, intended to be used for the manufacture of medicated feed (EC 2014). The transfer of traces of an active substance contained in a medicated feed to a non-target feed is referred to as 'carry-over'. 'Cross-contamination' is the contamination that results from the transfer of any unintended substance in feed (EC 2014). Feeding antimicrobial contaminated feeds may lead to unintended low dosage medication of the animals (Filippitzi *et al.* 2016, McEvoy 2002). Carry-over of antimicrobials contained in medicated feed should therefore be avoided or kept as low as possible by applying good manufacturing practice and the ALARA (As Low As Reasonably Achievable) principle (EC 2014).

2.2 ROUTES OF CROSS-CONTAMINATION AND RISK ASSESSMENT

The occurrence and levels of cross-contamination with antimicrobials and other veterinary drugs during feed processing depends on various factors including human error, production practices and handling procedures in the feed mill, during transport and at the farm (McEvoy 2002). The feed batches obtained just after production of a medicated feed are prone to cross-contamination (Borras *et al.* 2011). These batches are generally referred to as flush batches. The level of carry-over is mostly expressed as the

percentage of the veterinary drug (or additive) from a feed batch which ends up in the next feed batch (Stolker *et al.* 2013).

The specific properties of the drug and/or medicated premix (e.g., adhesive strength, particle size, and electrostatic properties) affect how and at which level cross-contamination occurs. Especially the electrostatic properties of drugs in powder form can aggravate the problem because of the difficult purging of the equipment between batches. The use of less electrostatic granular formulations (premixes) can thus reduce carry-over (McEvoy 2002).

Retention of residual quantities of medicated feed at various points along the production line may also be caused by technical factors such as fast production lines, deficiencies in plant layout and worn equipment parts (especially lifting screws and elevators) (EC 2014, Vukmirović *et al.* 2010). Cross-contamination may occur in a single piece of technological equipment, or may result from a combination of carry-over throughout the entire mixing process, manipulation and storage operations at the feed mill (Borras *et al.* 2011, McEvoy 2002). Finally, cross-contamination is a risk not solely concerning the feed mill, as it can also occur during transport and delivery and at farm level, during storage and distribution (Filippitzi *et al.* 2016, McEvoy 2002).

According to the ESVAC report (EMA 2016), antimicrobial premixes were the biggest-selling antimicrobial veterinary medicinal product for food-producing animals in the EU and accounted for 42.1% of the overall sales in 2014. There is, however, a large variation between the different European countries, for example regarding the preference of antimicrobial oral powders versus premixes. Although this high consumption of antimicrobial medicated feed suggests a substantial risk for cross-contamination, quantitative information on the estimated impact of the production, delivery and storage of antimicrobial medicated feed on cross-contamination is

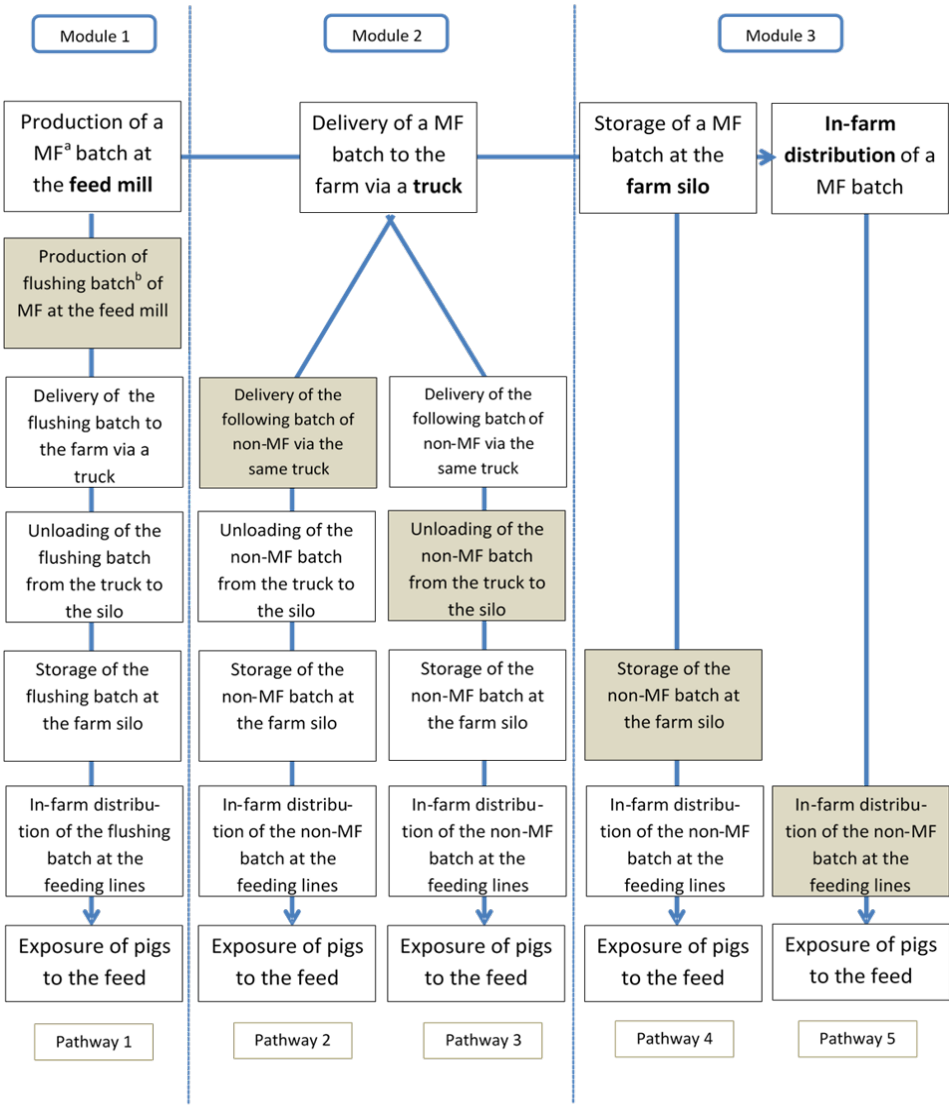


Figure 5. Flowchart of the model that estimates the percentages of cross-contaminated feed produced in a certain country per year: (a) MF stands for medicated feed; (b) although usually three flushing batches are produced after MF, the model took into consideration only the first flushing batch, since they contain considerably higher levels of antimicrobial residues than the second and the third. Adopted from Filippitzi et al. (2016).

very limited in the literature. Filippitzi and co-authors (2016) attempted to assess this impact quantitatively and examined all possible pathways of exposure of animals to feed which is cross-contaminated with traces of antimicrobials from previously produced, transported and stored medicated feed. The study initially focussed on medicated feed in Belgian pig production, but is applicable to a broader extent of cases such as other countries or animal species. This mathematical model, based on the flowchart in **Figure 5**, made it possible to estimate the percentages of cross-contaminated feed produced in a certain country per year. It should however be mentioned that no contamination levels were included in the model, because of the current lack of data.

2.3 CONSEQUENCES OF CROSS-CONTAMINATION WITH ANTIMICROBIALS

One of the reasons why cross-contamination of non-target feed with residues of antimicrobial compounds causes concerns is the potential for selection and dissemination of resistance in both commensal and pathogenic bacteria (Andersson *et al.* 2012, Chantziaras *et al.* 2014, EC 2014, Laxminarayan *et al.* 2013), as well as the potential selective pressure in the environment. Besides resistant pathogenic bacteria, commensal bacteria of animal origin may constitute a large reservoir of resistance genes. This reservoir provides multiple and complex pathways through which resistance genes can make their way over time into human pathogens via food, water and sludge and manure applied as fertilizer (Marshall *et al.* 2011).

Currently, there are no studies available that have assessed the impact of cross-contaminated feed on resistance selection in the intestinal microbiota. First of all, the antimicrobial concentrations in the gut that result from the consumption of feed containing low dosages of antimicrobials still need to be determined. To date there are only few studies available that

describe antimicrobial concentrations in intestinal content (Burch 2005, Burch *et al.* 2008, De Smet *et al.* 2017, Devreese *et al.* 2014), but these do not include low dosages as encountered by cross-contamination levels.

Inspired by the One Health concept, more and more researchers now also pay attention to aquaculture, sludge, freshwater and waste water as prime sites for gene exchange (Kim *et al.* 2014, Sanderson *et al.* 2016, Zhang *et al.* 2015). Antimicrobial residues can enter the environment directly via residues of antimicrobial medicated feed, e.g. in farm dust or via faeces and urine, which can be used as fertilizer on fields. It has been estimated that 25 to 75% of antibiotics used in food-producing animals are largely unmetabolized excreted, suggesting that even cross-contamination levels of antimicrobials in feed can cause considerable contamination of the environment with antimicrobials (Chee-Sanford *et al.* 2001, Hamscher *et al.* 2003).

2.4 MEDICATED FEED LEGISLATION

As stated above, the quality of a treatment via medicated feed is correlated with the level of manufacture standards and feed intake. Hence, the manufacture, marketing and use of medicated feed in the European Union is currently strictly regulated by the following Directives and Regulations (EC 2014).

Council Directive 90/167/EEC (Council of the European Communities 1990) constitutes the European Union's regulatory framework for the manufacture, placing on the market and use of medicated feed. The authorisation for use of veterinary medicinal products in feed, the manufacture, distribution, advertising and supervision of those veterinary medicinal products are governed by Directive 2001/82/EC of the European Parliament and of the Council (EP 2005a).

Medicated feed as a type of feed falls within the scope of Regulation (EC) No 183/2005 (EP 2005b), Regulation (EC) No 767/2009 (EP 2009) and Regulation (EC) No 1831/2003 (EP 2003) of the European Parliament and of the Council and of Directive 2002/32/EC of the European Parliament and of the Council (EP 2002). These regulations lay down requirements for feed hygiene, placing on the market and use of feed, additives for use in animal nutrition and undesirable substances in animal feed, respectively.

With the aim to update and harmonise the current rules which date from 1990, the Commission adopted a proposal on 10 September 2014 for a regulation on the manufacture, placing on the market and use of medicated feed and repealing Council Directive 90/167/EEC (Council of the European Communities 1990), in which rules on carry-over and preventive use are proposed (EC 2014). A general limit of 1% of the active substance of the last produced batch of medicated feed would account for carry-over of antimicrobials, a limit of 3% would account for other active substances. Preventive use of antimicrobial medicated feed would be prohibited and criteria for homogeneity would be established (EC 2014).

The proposed maximum carry-over of 1% corresponds with the guidelines that were established in Belgium since 2013 in the covenant between the Federal Agency for the Safety of the Food Chain (FASFC) and Belgian Compound Feed Industry Association (BFA). This covenant states that, starting from Jan 1st 2014, carry-over of antimicrobials should not exceed 1% of the minimal allowed dose, except for carry-over of tiamulin fumarate and tilmicosin in pelleted rabbit feed, that should not exceed 2.5%. Moreover, this covenant banned the use of the main mixer, which adds the premix in the middle of the mixing line, for antimicrobials in feed mills. Instead, feed mills now only mix veterinary antimicrobial drugs with feed at the end of the production line. This should reduce cross-contamination considerably. Other measures established in the covenant state that the

Belgian compound industry should only accept prescriptions from farm veterinarians (since Jan 1st 2016) for fattening pigs not older than 15 weeks (since Jan 1st 2015) (BFA and FASFC 2013).

In Belgium , the product conditions, the marketing and use of medicated feed and the modalities for prescription are regulated by means of the Royal Decree of 21 December 2006 (Royal Decree 2016).

3 PROPERTIES OF FREQUENTLY USED ANTIMICROBIALS IN PREMIXES

As mentioned before, tetracyclines and especially doxycycline are very important in veterinary medicine: they are the most sold antimicrobial class in Belgium as well as in Europe. Moreover, they are frequently applied as premix formulations in feed medication. It has been reported that tetracyclines have a low to moderate oral bioavailability in pigs (Baert *et al.* 2000, Nielsen *et al.* 1996), but it is not known to which intestinal concentrations this property would lead. The low oral bioavailability could possibly result in relatively high concentrations in the intestinal content, even at low concentration in the feed caused by cross-contamination. This could in turn lead to resistance selection in the intestinal microbiota.

To determine the effect of the oral bioavailability on intestinal concentrations, it is interesting to compare the tetracyclines with another antimicrobial group that shows a high oral bioavailability in pigs, e.g. sulphonamides and trimethoprim (Baert *et al.* 2001). These antimicrobials are also frequently applied as feed medication, and are therefore also prone to cross-contamination. Comparing these two classes with a distinct difference in oral bioavailability, can give more complete information on the correlation between this pharmacokinetic property and the resulting intestinal concentrations.

Within the group of tetracyclines, chlortetracycline and doxycycline, with an oral availability in pigs of 6% (Nielsen *et al.* 1996) and 20% (Baert *et al.* 2000), respectively, are discussed below. Being a commonly sold formulation, sulphadiazine-trimethoprim was chosen as a representative of sulphonamides-trimethoprim.

3.1 DEFINITIONS

3.1.1 PHYSICOCHEMICAL PROPERTIES

Physicochemical properties such as molecular weight, acid dissociation constant (pKa) and lipophilicity are important factors that determine the pharmacokinetics of a drug. An increase of molecular weight will decrease the drug's ability to diffuse through a liquid medium. The acid dissociation constant of a drug is defined as the pH at which equal proportions of the drug exist in its ionised and unionised form. As mainly unionised compounds are considered to be able to penetrate biological membranes by passive diffusion, the pKa is an important parameter in pharmacokinetics (Martinez 1998b). The lipophilicity scale, expressed by the parameter log *P*, influences the disposition of the drug in different body compartments. The value of log *P* is used to define a drug as lipophilic (> 1) or hydrophilic (< 1) and is positively correlated with the volume of distribution and the ability to cross biological membranes (Grabowski *et al.* 2009, Martinez 1998b).

3.1.2 PHARMACOKINETICS/PHARMACODYNAMICS

The relationship between systemic antimicrobial drug exposure and its corresponding clinical and microbiological effects is referred to as '**pharmacokinetics/pharmacodynamics (PK/PD)**'.

Pharmacokinetics (PK) describe the time course of drug levels in body fluids as a result of absorption, distribution, metabolisation and elimination of a drug after administration (Levison *et al.* 2009). The pharmacokinetic properties of a drug, namely the extent of absorption (systemic availability), pattern distribution and rate of elimination are largely determined by the chemical nature of an antimicrobial drug and the related

physicochemical properties. Moreover, interspecies variation in bioavailability, clearance and volume of distribution can occur due to influences of factors such as genetic variability (breed effects), disease/stress, specific physiological conditions, hepatic and renal function, environment, food, gender and age (Martinez *et al.* 2010). **Bioavailability** is defined as the rate and extent to which the active drug ingredient or therapeutic moiety is absorbed from a drug product and becomes available at the site of drug action (Rescigno 1997). The **oral bioavailability** in particular is a measure of the rate and extent of a drug reaching the systemic circulation in its unchanged form through intestinal absorption (Hu *et al.* 2011). The **volume of distribution** is used as a measure of the extent to which a pharmaceutical compound distributes around the body (Yates *et al.* 2008). The volume of distribution of a specific body compartment is calculated as the ratio between the amount of a drug present in the body and its concentration in the compartment (Rescigno 1997). **Total body clearance**, the total effect of all elimination processes, can be defined as the amount of drug eliminated per unit of time (Rescigno 1997). It is expressed in terms of the volume of blood that is totally cleared of a drug per unit of time and can be determined in terms of total systemic clearance or in terms of clearance associated with a particular eliminating organ such as the kidney or the liver (Martinez 1998a). The **elimination half-life** is defined as the time needed for the plasma concentration, as well as the amount in the body, to decrease by 50% through the process of elimination (Desmond Baggot *et al.* 2013, Rescigno 1997).

The **pharmacodynamics (PD)** of an antimicrobial drug describe the relationship between its PK and the time course of the antimicrobial effects at the site of the infection (Levison *et al.* 2009). In other words, the PD of an antimicrobial drug describe the effect of the drug over time on the bacteria at the site of infection (Martinez *et al.* 2013). This effect is generally estimated based on the MIC (minimum inhibitory concentration) and PK

parameters of the antimicrobial, using different calculations depending on the antimicrobial type (dose- or time- dependent): (1) the percentage of the dosing interval for which the plasma concentration exceeds the MIC (%T/MIC); (2) the maximum plasma concentration (C_{max}) divided by the MIC (C_{max}/MIC); and (3) the area under the concentration versus time curve (AUC) divided by the MIC. Clinical trials help to establish whether these predicted concentrations/doses generate the desired clinical and microbiological outcome (Levison 2004, Martinez *et al.* 2013).

3.2 TETRACYCLINES

The tetracyclines are the most used class of antimicrobials in veterinary medicine (33.4% of total sales in Europe in 2014). They are first-line antimicrobials in food-producing animals, including aquaculture, honeybees and exotic animals. Moreover, tetracyclines are broad-spectrum antimicrobials with activity against Gram positive and Gram negative bacteria, mycoplasmas, some mycobacteria, most pathogenic alpha-proteobacteria, and several protozoan and filarial parasites (del Castillo 2013). Consequently tetracyclines are classified by the OIE as critically important antimicrobials for veterinary medicine (OIE 2015). An antimicrobial is classified as critically important when it meets two criteria: (1) in a questionnaire, more than 50% of the respondents identified the importance of the antimicrobial class and (2), the antimicrobial was identified as essential against specific infections, with a lack of sufficient therapeutic alternatives.

Tetracyclines are also used for growth promotion in food-producing animals but concerns on emerging resistance led to a withdrawal of tetracyclines from the list of authorized growth promoters in many European countries in 1972–1974 (Cogliani *et al.* 2011). However, many countries outside the EU including the US still allow the use of tetracyclines for growth promotion (Chopra *et al.* 2001, Sorensen *et al.* 2014). Although growth

promotors are still legal in the US, the FDA (Food and Drug Administration) established a 'Guideline for Industry' in 2013 which encourages pharmaceutical companies to no longer produce antimicrobial formulations intended for growth promotion. This guideline also states that antimicrobials should only be distributed under veterinary prescription (FDA 2013).

The first discovered tetracycline was chlortetracycline, which was isolated from *Streptomyces aureofaciens* by Duggar (Duggar 1948). Oxytetracycline, a secondary metabolite of *Streptomyces rimosus*, was discovered shortly afterwards (Finlay *et al.* 1950). Other naturally occurring first generation tetracyclines were identified further on, i.a. tetracycline and demethylchlortetracycline (Nguyen *et al.* 2014). Second generation tetracyclines such as doxycycline and minocycline, were developed using semisynthetic approaches (Nguyen *et al.* 2014). These compounds showed improved PK properties, increased antimicrobial potency and decreased toxicity. In the 1980s, emerging antimicrobial resistance renewed interest in the development of a third generation of tetracyclines, the semisynthetic glycylcyclines of which tigecycline is currently the only representative (Chopra *et al.* 2001, Nguyen *et al.* 2014). Tigecycline shows activity against both efflux and ribosomal protection mediated tetracycline resistance, but is not authorized for use in animals (EMA 2013, Sum *et al.* 1994).

3.2.1 PHYSICOCHEMICAL PROPERTIES

The name 'tetracyclines' is based on the core structure typical for these antimicrobials, comprising four aromatic rings: 2-naphtacene carboxyamide (Stephens *et al.* 1952). The chemical structures of some first, second and third generation tetracyclines are given in **Figure 6**, together with the naphtacene core.

All tetracyclines are amphoteric molecules and are ionized at all pH values. The proportions of zwitterions, cations and anions that are

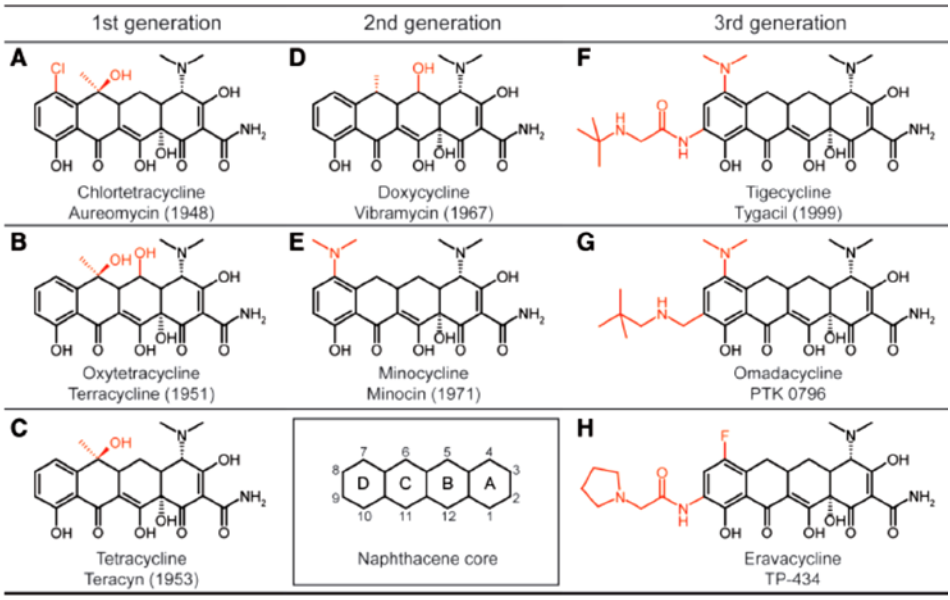


Figure 6. Chemical structures of tetracyclines. Chemical structures of the first generation tetracyclines (A) chlortetracycline, (B) oxytetracycline and (C) tetracycline, of the second generation tetracyclines (D) doxycycline and (E) minocycline, and of the third generation tetracyclines (F) tigecycline, (G) omadacycline and (H) eravacycline. The year the antibiotic was discovered/reported is indicated in parentheses. The inset of the DCBA naphthacene core provides the carbon atom assignments for rings A–D. Adopted from Nguyen *et al.* (2014).

present in solution depend on the pH of the medium. The null net charge of the zwitterionic form, which predominates at pH values between 4 and 7, favours its passage across cell membranes.

Tetracyclines are hardly soluble in water. Therefore, they are administered orally or parentally as acid or basic salts. Except for chlortetracycline, that degrades at a rate increasing with pH, the tetracyclines are fairly stable at physiological pH values (del Castillo 2013). In aqueous solution, tetracyclines are sensitive to U.V. degradation (Gómez-Pacheco *et al.* 2012). Tet-

racycline-HCl in dry state is stable when stored at room temperature, regardless of exposure to light (Wu *et al.* 2005). Tetracyclines are strong chelators, and chelation with multivalent metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}) influences their PK properties, oral bioavailability and antimicrobial activity (del Castillo 2013).

The chemical formula, molecular weight, pKa and log *P* of chlortetracycline and doxycycline, the two tetracyclines that were used in the studies described in this thesis, are given in **Table 2**.

Table 2. Physicochemical properties of chlortetracycline and doxycycline

	Chlortetracycline[a]	Doxycycline[b]
Chemical formula	$C_{22}H_{23}ClN_2O_8$	$C_{22}H_{24}N_2O_8$
Molecular weight (g/mol)	515.3	444.44
pKa	3.3, 7.4, 9.3	3.5, 7.7, 9.5
log *P*	-0.62	1.777

[a] Qiang *et al.* (2004), PHYSPROP (2017)
[b] Vandenberghe *et al.* (2012b)

3.2.2 PHARMACOKINETIC AND PHARMACODYNAMIC PROPERTIES

The PK properties of tetracyclines are influenced by several factors such as their molecular size, partition coefficient (log *P*), plasma protein binding, the acidity of biological mediums, their exposure to multivalent cations and the expression level of ABC transporter proteins such as P-glycoprotein in the cell membranes of the target tissue (del Castillo 2013). The four key PK properties (bioavailability, volume of distribution, clearance and elimination half-life) of chlortetracycline and doxycycline are given in **Table 3**.

Table 3. Estimates of the pharmacokinetic properties of chlortetracycline and doxycycline when orally administered in non-fasted pigs

	Chlortetracycline[a]	Doxycycline[b]
Oral bioavailability (%)	6-17.9	21.2-50
Elimination half-life (h)	2.77-4.62	2.9
Volume of distribution (l/kg BW)	1.29-2.34	0.97
Clearance (l/h·kg BW)	0.20-0.467	0.173

[a] del Castillo *et al.* (1998), Nielsen *et al.* (1996)

[b] Baert *et al.* (2000), Sanders *et al.* (1996). BW: body weight

The bioavailability of oral administered tetracyclines depends on the partition coefficient of the tetracycline and can be negatively influenced by the presence of food particles and multivalent cations (Baert *et al.* 2000, del Castillo 2013). The oral bioavailability of doxycycline in pigs seems to vary greatly, which may partly be due to differences in study design, e.g. the prandial state of the animals. However, even within the same study, wide variations between individual animals (21.2–50%) can be found (Baert *et al.* 2000, Sanders *et al.* 1996). Also in other animal species, the oral bioavailability of doxycycline is rather low. This is in contrast with the oral bioavailability in humans, which is higher and does not seem to be affected by the presence of food (Baert *et al.* 2000).

The volumes of distribution of tetracyclines are variable because they differ in lipid solubility (Desmond Baggot *et al.* 2013). The distribution of tetracyclines is typically highest in richly perfused organs: kidney > liver ≥ lungs > blood = synovia > muscle. Because of their multivalent cation-chelating properties, tetracyclines are deposited easily in teeth and at sites of new bone formation, which can have toxicological consequences (del Castillo 2013). The volume of distribution of doxycycline in pigs is generally smaller than in other species (Baert *et al.* 2000).

Excretion of tetracyclines primarily happens by glomerular filtration, by biliary excretion (at an extent that correlates with their lipid solubility) and by intestinal excretion via P-glycoproteins. They undergo enterohepatic circulation: a large fraction of the drug excreted in the bile is reabsorbed from the intestines (del Castillo 2013).Once arrived at the site of infection, tetracyclines exert their broad-spectrum antibacterial activity by protein synthesis inhibition (**Figure 7**). This results in a bacteriostatic effect. Moreover, a time-dependent and dose-dependent (see 4.2,) bacteriocidic effect has been proven for e.g. doxycycline (Cunha *et al.* 2000). The antibacterial potency of the tetracyclines positively correlates with lipophilicity, as this determines the possibilities for intracellular diffusion, and thus varies between different tetracyclines. The semisynthetic derivatives (e.g. doxy-

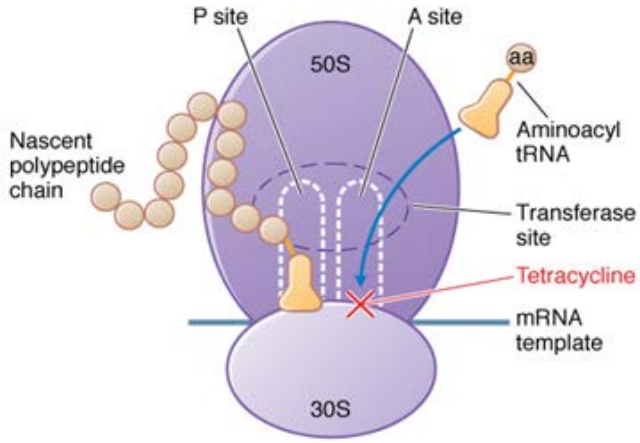


Figure 7. Tetracycline mechanism of action. Tetracyclines bind at the decoding centre of the small subunit of the ribosome, i.e. where the codon of the mRNA (messenger ribonucleic acid) is recognized by the anticodon of the tRNA. More specifically, rings C and D of the tetracycline sterically clash with the first nucleotide of the anticodon of the tRNA (transfer RNA) that interacts with the third base of the A-site codon of the mRNA. This inhibits the process of peptide synthesis (Nguyen et al. 2014).
Figure adopted from http://www.antibiotics-info.org.

cycline) are most active, followed by the chlorinated tetracyclines (e.g. chlortetracycline) and finally oxytetracycline and tetracycline (del Castillo 2013).

3.3 SULPHONAMIDES-TRIMETHOPRIM

Sulphonamides were the first clinically successful broad-spectrum antimicrobials, produced in 1935 in Germany (Giguère 2013). They inhibit bacteria, toxoplasma and other protozoal organisms such as coccidia. A synergistic action is obtained when combined with antibacterial diaminopyrimidines such as trimethoprim (Prescott 2013). Sulphonamides or sulphonamide-diaminopyrimidine combinations are classified as critically important for the treatment of a wide range of bacterial, coccidial and protozoal infections in a wide range of animal species (OIE 2015).

In the US, trimethoprim/sulphonamide combinations are not approved for oral use in animals (Burch 2013).

3.3.1 PHYSICOCHEMICAL PROPERTIES

The sulphonamides are derivatives of sulphanilamide, which contains the structural prerequisites for antimicrobial activity (Prescott 2013). The structures of sulphanilamide and sulphadiazine, the sulphonamide that will be discussed in this thesis, are given in **Figure 8**.

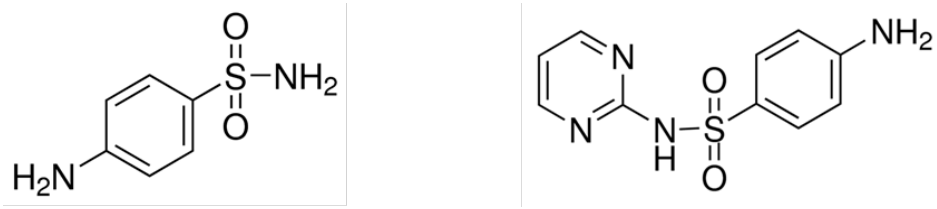


Figure 8. Chemical structures of sulfanilamide (left) and sulfadiazine (right)

Sulphonamides are weak acids with pKa values ranging from 5.0 to 10.4. The non-ionized form, that exists predominantly in biological fluids of pH lower than their pKa, diffuses through cell membranes and penetrates cellular barriers (Prescott 2013). It has been shown that sulphonamides undergo photodegradation, but the degradation rates of various sulphonamides under the same conditions are different (Zessel *et al.* 2014). Trimethoprim (**Figure 9**), a synthetic drug, is a weak base with a pKa of about 7.6 and is poorly soluble in water (Prescott 2013). In contrast to sulphonamides, trimethoprim has been shown to be stable following natural U.V. exposure (Luo *et al.* 2012).

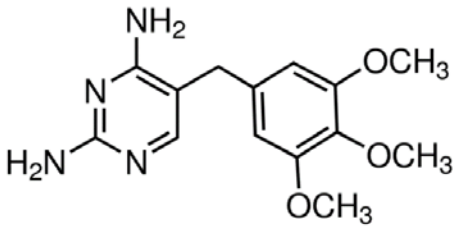


Figure 9. Chemical structure of trimethoprim

Important physicochemical properties of sulphadiazine and trimethoprim are given in **Table 4**.

Table 4. Physicochemical properties of sulphadiazine and trimethoprim

	Sulphadiazine[a]	Trimethoprim[b]
Chemical formula	$C_{10}H_{10}N_4O_2S$	$C_{14}H_{18}N_4O_3$
Molecular weight (g/mol)	250.25	290.32
pKa	6.81, 1.64, 6.36	7.12
Log *P*	-0.09, -0.074, -0.34	0.91

[a] Vandenberge *et al.* (2012b).
[b] PHYSPROP (2017). pKa: acid dissociation constant; log *P*: lipophilicity scale.

3.3.2 PHARMACOKINETICS AND PHARMACODYNAMIC PROPERTIES

Pharmacokinetic properties vary widely among the individual sulphonamides. The optimal plasma ratio of trimethoprim/sulphonamide is usually 1:20 because trimethoprim is 20 times more active than sulphonamide (Bushby 1980). To achieve this ratio, combination formulations normally contain trimethoprim and a sulphonamide in a 1:5 ratio (Prescott 2013).

Important PK properties of oral administered sulphadiazine and trimethoprim in swine (oral bioavailability, elimination half-life, volume of distribution and clearance) are summarized in **Table 5**.

Table 5. Estimates of the pharmacokinetic properties of sulphadiazine and trimethoprim when orally administrated in non-fasted pigs

	Sulphadiazine[a]	Trimethoprim[b]
Oral bioavailability (%)	85-90	73-92
Elimination half-life (h)	2.4-8	2.73
Volume of distribution (l/kg BW)	0.55-0.83	2.24
Clearance	0.78-2.3 ml/min.kg BW	0.54 l/h.kg BW

[a] Baert *et al.* (2001), Nielsen *et al.* (1994), Witt (2006).
[b] Baert *et al.* (2001), Nielsen *et al* (1994). BW: body weight.

Most sulphonamides have a good to very good oral bioavailability. The high oral bioavailability of sulphadiazine in pigs is not affected by the presence of feed (Baert *et al.* 2001). Trimethoprim is also rapidly absorbed and to a large extent after oral administration, except in ruminants, where it undergoes microbial degradation in the rumen (Boothe 2016, Prescott 2013).

Most of the sulphonamides and trimethoprim are well distributed in all tissues and body fluids, including synovial and cerebrospinal fluids (Prescott 2013).

Sulphonamides are eliminated by renal excretion and biotransformation. The renal excretion includes glomerular filtration of free drug in the plasma, active carrier-mediated proximal tubular secretion of ionized unchanged drug metabolites, and passive reabsorption of non-ionized drug from distal tubular fluid. The pKa of the sulphonamide and the pH of the fluid in the distal tubules determines the extent to which it is reabsorbed (Prescott 2013). The elimination half-lives are relatively long, due to extensive binding to plasma albumin and pH-dependent passive reabsorption from acidic distal renal tubule fluid (Prescott 2013). Some very water-soluble sulphonamides, e.g. sulphisoxazole and sulphasomidine, are rapidly eliminated via renal excretion, mostly in unchanged form, and are therefore primarily used to treat urinary tract infections (Boothe 2016). Biotransformation of sulphonamides through acetylation, glucuronide conjugation and aromatic hydroxylation occurs mainly in the liver (Prescott 2013). Trimethoprim is mainly eliminated by hepatic metabolisation (oxidation followed by conjugation reactions) and the elimination half-life depends on the animal species. The elimination half-lives of trimethoprim and the combined sulphonamide are often different, but the combination is normally clinically effective because of the relatively broad range over which synergism occurs (Prescott 2013). In pigs, trimethoprim and sulphadiazine have similar half-lives (**Table 5**), which favours the desired plasma ratio.

Sulphonamides-trimethoprim combinations exhibit their synergistic antibacterial effect by inhibiting sequential steps of the folic acid production and thus of the purines required for DNA (deoxyribonucleic acid) synthesis (Prescott 2013). Sulphonamides compete with para-aminobenzoic acid (PABA) for the enzyme dihydropteroate synthetase, thus preventing PABA from incorporation into the folic acid molecule. Consequently, the

bacteriostatic action can be reversed by an excess of PABA, e.g. in case of presence of tissue exudates and necrotic tissue. The selective bacteriostatic action is caused by the different sources of folic acid between mammalian and bacterial cells: mammalian cells use preformed folic acid, whereas susceptible microorganisms must synthesize folic acid (Prescott 2013). Diaminopyrimidines such as trimethoprim interfere with the synthesis of tetrahydrofolic acid from dihydrofolate by inhibiting the enzyme dihydrofolate reductase. Here, the selective antibacterial effect is caused by the greater affinity for the bacterial rather than the mammalian dihydrofolate reductase (Prescott 2013).

The synergistic effect of sulphonamides and trimethoprim on the folic acid metabolism is shown schematically in **Figure 10**.

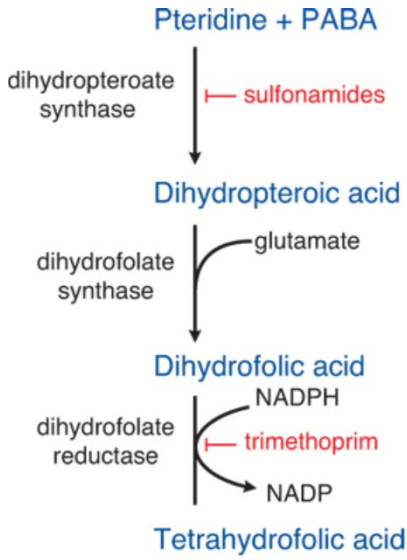


Figure 10. Steps in folic acid metabolism blocked by sulphonamides and trimethoprim. Co-administration of a sulphonamide and trimethoprim introduces sequential blocks in the biosynthetic pathway for tetrahydrofolate. The combination is much more effective than either agent alone. PABA: para-aminobenzoic acid; NADP(H): nicotinamide adenine dinucleotide phosphate. Adopted from Brunton *et al.* (2007).

4 ANTIMICROBIAL RESISTANCE

4.1 INTRODUCTION

The availability and use of antimicrobial drugs had a huge impact on the practice of human and animal medicine. Bacterial infections that used to be lethal are now treatable, and the use of antimicrobial drugs has advanced global public health, animal health, and food safety and security. Unfortunately, antimicrobials are not infallible. Bacteria can be intrinsically resistant to certain antimicrobials, which means they have the ability to resist the action of a particular antimicrobial as a result of inherent structural or functional characteristics. Further, bacteria can acquire resistance to antimicrobials via mutations in chromosomal genes and by horizontal gene transfer (Blair *et al.* 2015). Bacteria must have acquired different resistance mechanisms since a long time. Natural antimicrobials are substances that are produced by fungi or soil bacteria and as such there has been contact with these substances (and other antimicrobial products as metals) long before their clinical use (Schwarz *et al.* 2001a). The current high use of antimicrobial products has dramatically contributed to the selection of antimicrobial resistant organisms, which pose an extraordinary threat to human and animal health, and to the world's ecosystem (OIE 2016). It is estimated that each year, drug-resistant infections result in at least 25,000 deaths and the cost associated with antimicrobial resistance in the EU is estimated at 1.5 billion euro through increased healthcare costs and losses in productivity (EC 2015). Hence, antimicrobial resistance is listed as one of the greatest threats to human health worldwide (Blair *et al.* 2015). To combat the spread of antimicrobial resistance, the WHO has published the 'Global Action Plan on Antimicrobial Resistance' in collaboration with the Food and Agriculture Organization of the United Nations

(FAO) and OIE (WHO 2015). Similarly, the EU adopted a new One Health Action Plan against antimicrobial resistance in 2017. The new plan contains concrete actions with EU added value that the Commission will develop and strengthen as appropriate in the coming years for a more integrated, comprehensive and effective approach to combating antimicrobial resistance.

4.2 MECHANISM OF ACTION OF ANTIMICROBIALS

Antimicrobials act selectively on vital microbial functions without compromising the host functions, and different classes of antimicrobials act on different metabolic pathways. Nevertheless, antimicrobials can cause adverse effects due to direct effects on host cells, hypersensitivity, drug interactions, changes in microbiota and microbial lysis (Mandell *et al.* 2001).

Antimicrobials can act bacteriostatic and/or bactericidal. Bacteriostatic antimicrobials only inhibit the growth or multiplication of bacteria, whereas bactericidal antimicrobials kill the bacteria. Consequently, for the bacteriostatic antimicrobials the combination with the host immune system is of much more importance than for the bactericidal antibiotics to eliminate the bacterial infection. However, this classification in bacteriostatic and bactericidal antimicrobial is an approximation, depending on both the drug concentration at the site of infection and the microorganism involved. The distinction between bactericidal and bacteriostatic is made based on the MIC (Minimum Inhibitory Concentration) and the MBC (Mimimum Bactericidal Concentration) of the drug. The MIC is defined as the lowest concentration of an antimicrobial agent (in mg/l) that, under defined *in vitro* conditions, prevents the appearance of visible growth of a microorganism within a defined period of time (ISO 20776-1:2006). Generally, the antimicrobial is considered bactericidal when the MBC does not exceed four times the MIC (Giguère 2013). Furthermore, antimicrobials

can be classified as exerting either time-dependent or concentration-dependent activity. In the first group, the time that serum concentrations exceed the MIC determines the efficacy of the treatment, whereas in the second group, the efficacy increases with increasing serum concentrations (Giguère 2013).

The modes of action of antimicrobials can be subdivided in four major categories: inhibition of the cell wall synthesis, inhibition of ribosome function, inhibition of nucleic acid synthesis and alteration of cell membrane function (Giguère 2013, Levinson 2014).

4.3 MECHANISMS OF ANTIMICROBIAL RESISTANCE

4.3.1 OVERVIEW OF RESISTANCE MECHANISMS

As mentioned above, some bacteria are intrinsically resistant against certain antimicrobials. Besides that, antimicrobial pressure has forced susceptible bacteria to escape the inhibitory action of antimicrobial agents, by acquiring different resistance mechanisms.

Four main mechanisms of resistance have been identified:

— **Enzymatic inactivation** of the antimicrobial. Antimicrobials can be enzymatically modified or degraded before they reach the target site. An important example is the resistance against β-lactam antibiotics through production of β-lactamases (Smet *et al.* 2010).
— **Alternative metabolic pathways**. Bacteria can 'bypass' the inhibited metabolic pathway e.g. by overproducing the antibiotic target or production of a modified enzyme. This mechanism is used in case of sulphonamides and trimethoprim, by hyperproduction of PABA or production of an insensitive dihydropteroate enzyme (Munita *et al.* 2016, Prescott 2013).

— **Alteration of the target**. A mutation or post-transcriptional or post-translational modification of the antimicrobial agent's target, or the acquisition of an alternative less susceptible target that can replace the function of the susceptible target can reduce the binding of the antimicrobial or have the function of the target being replaced. This mechanism can cause resistance to for example rifampicin and quinolones (Ruiz 2003, Tupin *et al.* 2010) or resistance to tetracyclines (ribosomal protection mechanisms) (Nguyen *et al.* 2014).
— **Lowering the internal concentration** of the antimicrobial. This can be achieved by reduced uptake or by active efflux of the antimicrobial. Efflux mechanisms mediate resistance against a wide variety of antimicrobials, e.g. tetracyclines (Blanco *et al.* 2016).

4.3.2 TETRACYCLINE RESISTANCE MECHANISMS

It is not known if intestinal concentrations of doxycycline caused by cross-contaminated feed can exert a selective effect in the intestinal microbiota of pigs. To investigate this, a thorough knowledge of tetracycline resistance mechanisms and their transfer is necessary.

Antimicrobial resistance can occur due to mutations or the acquisition of resistance genes, but tetracycline resistance has been shown to be caused mainly by the latter mechanism. The prevalence of resistance against tetracyclines was very low when they were first introduced for clinical, veterinary and agricultural use, but since then many commensal and pathogenic bacteria have acquired resistance genes (Chopra *et al.* 2001). One of the reasons for the rapid increase of the prevalence of tetracycline resistance is the location of the resistance genes on highly mobile genetic elements, such as plasmids and transposons, resulting in efficient horizontal gene transfer (Thaker *et al.* 2010).

There are four main mechanisms of tetracycline resistance: active efflux, ribosomal protection, enzymatic inactivation and rRNA mutations. Of these four, active efflux is the most prevalent one, followed closely by ribosomal protection. Less prevalent mechanisms are the monooxygenases that promote degradation of tetracyclines and mutations within the 16S rRNA that reduce the binding affinity of the drug for the ribosome (Nguyen *et al.* 2014).

Efflux pumps

Thirty three different efflux pump genes (**Table 6**) have been identified to date (Roberts 2016). These genes fall into seven defined phylogenetic groups (Guillaume *et al.* 2004). The group 1 H^+ antiporters is by far the largest group and comprises the most prevalent tetracycline-resistance determinant in Gram negative bacteria. The group 1 efflux pumps, like TetA, show high homology with the major facilitator superfamily of secondary active transporters. These efflux pumps exchange a proton for the tetracycline molecule against a concentration gradient (Blanco *et al.* 2016). Tetracycline efflux pumps confer resistance to tetracycline, but many of them are less effective against second generation tetracyclines (doxycycline, minocycline) and confer little to no resistance to the third generation glycylcycline, tigecycline (Chopra *et al.* 2001, Grossman *et al.* 2012).

Because the expression of antibiotic resistance genes can be associated with a fitness cost or physiological impairment, many bacteria regulate the expression of their resistance genes by different mechanisms (translational attenuation, transcriptional attenuation, translational coupling) (Chopra *et al.* 2001). Regulation of tetracycline resistance genes often happens by negative control by a TetR repressor protein. This protein binds to two tandemly orientated *tet* operators to block transcription of the efflux pump in the absence of tetracycline. When tetracycline enters the cell, it binds to

Table 6. Mechanism of tetracycline resistance for characterized *tet* and *otr* genes

Efflux (33)	Ribosomal Protection (12)	Enzymatic Inactivation (13)
tet(A), *tet*(B), *tet*(C),	*tet*(M), *tet*(O), *tet*(S),	*tet*(X)
tet(D), *tet*(E), *tet*(59)	*tet*(W), *tet*(32),	*tet*(37)
tet(G), *tet*(H), *tet*(J),	*tet*(Q), *tet*(T), *tet*(36)	*tet*(34)
tet(V), *tet*(Y)	*otr*(A), *tetB*(P)b, *tet*	*tet*(47), *tet*(48), *tet*(49),
tet(Z), *tet*(30), *tet*(31),	*tet*(44)	*tet*(50), *tet*(51), *tet*(52),
tet(33), *tet*(57)		*tet*(53), *tet*(54), *tet*(55),
tet(35)		*tet*(56)
tet(39), *tet*(41)		
tet(K), *tet*(L), *tet*(38),		
tet(45), *tet*(58)		
tetA(P), *tet*(40)		
otr(B), *otr*(C)		
tcr		
tet(42)		
tet(43)		
*tet*AB(46)		
*tet*AB(60)		

Adopted from Roberts (2016) https://faculty.washington.edu/marilynr/tetweb1.pdf

TetR, which in its turn dissociates from the *tet* operator (**Figure 11**). As such, transcription and expression of the efflux pump is induced (Moller *et al.* 2016, Saenger *et al.* 2000).

Tetracycline efflux pumps are frequently detected in bacterial isolates from pigs, e.g. *E. coli*, *Salmonella spp.*, *Streptococcus suis*, *Enterococcus spp.*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, etc. (Blanco *et al.* 2006, Chen *et al.* 2013, Lancashire *et al.* 2005, Prüller *et al.* 2015, Seputiene *et al.* 2012).

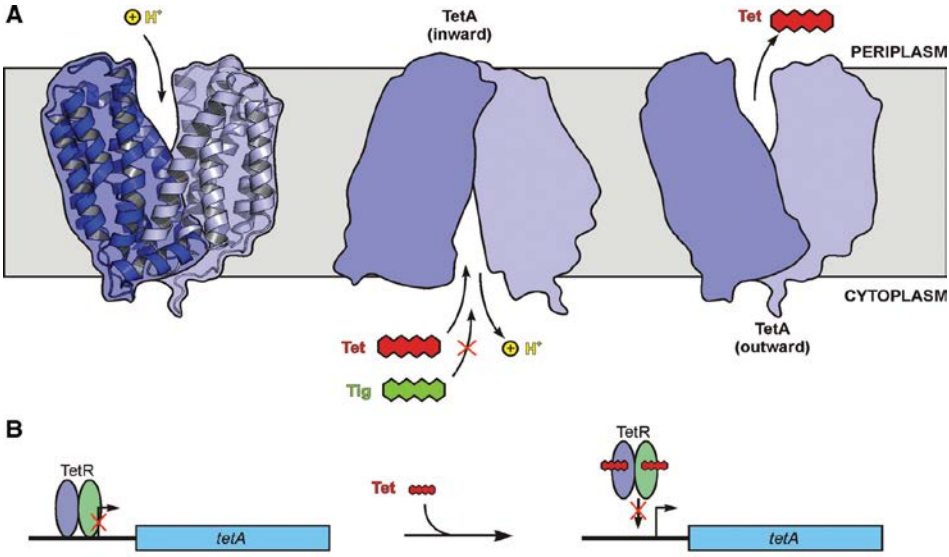


Figure 11. TetR-mediated regulation of the tetracycline resistance TetA efflux pump. **(A)** Schematic for mechanism of action of efflux pump TetA, illustrating that efflux of tetracycline (but not tigecycline) is coupled to proton transport. **(B)** Schematic for TetR-mediated regulation of TetA, illustrating that tetracycline binding to the TetR homodimer leads to activation of transcription of the *tet*(A) gene. Adopted from Nguyen et al. (2014).

Ribosomal protection

Ribosomal protection proteins (RPPs) are a widely distributed class of tetracycline resistance determinants and are part of the translation factor superfamily of the GTPases (Connell *et al.* 2003, Leipe *et al.* 2002, Thaker *et al.* 2010). These cytoplasmic proteins share considerable homology with the ribosomal elongation factors EF-G and EF-Tu (Sanchez-Pescador *et al.* 1988). Phylogenetic analysis shows that RPPs probably have been derived from *otr*A, which confers tetracycline resistance in *Streptomycus rimosus*, the natural producer of oxytetracycline (Doyle *et al.* 1991). Until now, 12 different RPP genes have been identified (**Table 6**) of which *tet*(M) and *tet*(O) are the most prevalent (Roberts 2016, Thaker *et al.* 2010). The *tet*(M) genes occur in a wide variety of Gram positive and Gram negative bacteria.

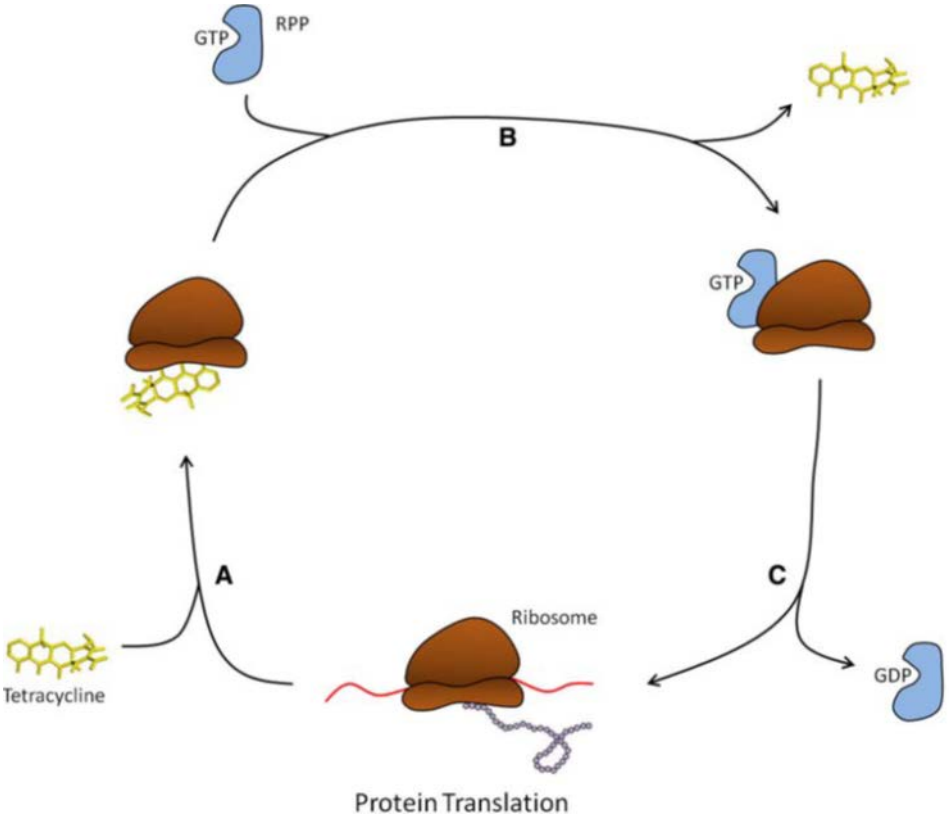


Figure 12. Ribosomal protection protein (RPP)-mediated tetracycline resistance. **(A)** Tetracycline binds the ribosome at the apex of the A-site which in turn sterically blocks the aminoacyl-tRNA binding site and inhibits protein synthesis. **(B)** When bound by tetracycline, RPPs along with bound GTP will associate with the ribosome which results in tetracycline release from the A-site. **(C)** Upon tetracycline release, GTP is hydrolyzed and the RPP subsequently dissociates from the ribosome which restores protein synthesis. tRNA = transfer RNA; GTP = guanosinetriphosphate. Adopted from Thaker *et al.* (2010).

This gene has been found commonly on conjugative transposons, which exhibit an extremely broad host range (Roberts 1996). The RPPs confer tetracycline resistance by releasing tetracycline from the ribosome and thereby freeing the ribosome from the inhibitory effects of the drug, such that aminoacyl-tRNA can bind to the A-site of the ribosome and protein synthesis can continue (**Figure 12**) (Connell *et al.* 2003).

Various porcine bacterial isolates have been shown to carry RPP genes, such as *Enterococcus spp.*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, etc. (Blanco *et al.* 2006, Chen *et al.* 2013, Seputiene *et al.* 2012).

Enzymatic inactivation

Enzymatic inactivation of tetracycline seems relatively rare compared to other natural antibiotic classes such as the β-lactams and aminoglycosides, where drug inactivation and modification is the predominant resistance mechanism (Forsberg *et al.* 2015, Thaker *et al.* 2010). To date, 13 different genes have been found to encode for inactivation of tetracyclines (**Table 6**), of which ten – a novel family called 'tetracycline destructases' – have been discovered only recently (Forsberg *et al.* 2015, Roberts 2016). Of the three initial detected enzymes (Tet(X), Tet(34), and Tet(37)), only TetX has been studied in detail. It was shown to be a flavoprotein monooxygenase that inactivates tetracycline antibiotics by monohydroxylation and reduces the binding of the modified molecule to magnesium, essential for its binding to the ribosome. This is then followed by spontaneous, non-enzymatic breakdown (Moore *et al.* 2005, Thaker *et al.* 2010, Volkers *et al.* 2011, Yang *et al.* 2004). Tet(X) confers resistance to all classes of tetracyclines including minocycline and tigecycline (**Figure 13**). TetX has been discovered originally in *Bacteroides fragilis* but has not been detected in porcine isolates so far (Speer *et al.* 1989).

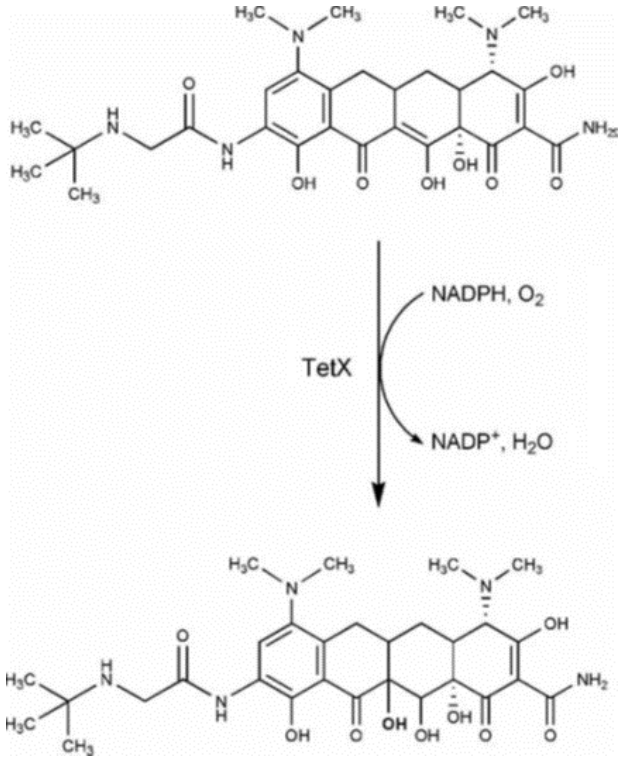


Figure 13. TetX mediated tigecycline inactivation. The enzymatic inactivation of tigecycline is mediated by TetX, a flavin-dependent monooxygenase. In the presence of oxygen, TetX catalyze the regiospecific hydroxylation of tigecycline at position 11a producing 11a-hydroxytigecycline. Adopted from Thaker et al. (2010).

Other mechanisms of tetracycline resistance

The other, less prevalent mechanisms of tetracycline resistance are reduced antibiotic permeability and target modification (Thaker *et al.* 2010).

The **permeability** of the outer membrane of Gram negative bacteria can be reduced by downregulating the synthesis of the OmpF porin, by which tetracycline crosses the outer membrane, resulting in less influx (Cohen *et al.* 1988).

Bacteria can escape antimicrobial action via **target modification**, for example the mutation in the 16S rRNA. Tetracycline has a decreased affinity for ribosomes bearing these so called G1058C mutations compared to wild-type ribosomes. These mutations have been found in *Helicobacter pylori* (Nonaka *et al.* 2005).

4.4 SELECTION AND TRANSFER OF ANTIMICROBIAL RESISTANCE

4.4.1 RESISTANCE SELECTION

Introduction

Although it is well known that antimicrobial use selects for antimicrobial resistance, it has been debated how and where exactly the resistance arises and is enriched (Holmes *et al.* 2016). Even though many (micro)organisms naturally produce antimicrobial substances, little evidence exists to suggest that this contributes significantly to the selection of antimicrobial-resistant microorganisms in their native environment (Martinez 2009). Nevertheless, the resistance mechanisms are shown to be ancient (clusters of) genes, often phylogenetically related to genes encoded by the antibiotic producing micro-organism. The extensive human, veterinary and agricultural use of antimicrobials is considered as the key driver of antimicrobial resistance selection (Bell *et al.* 2014, Laxminarayan *et al.* 2013).

Many factors can influence the speed of resistance selection, but antimicrobial exposure dynamics are considered a main factor. Clear correlations between antimicrobial use and resistance development have been reported. The countries that use most antimicrobials, generally also face the highest burden of resistance in clinically important bacteria (Bell *et al.* 2014, Chantziaras *et al.* 2014, JIACRA 2015).

Next to the exposure to antimicrobials there are several other factors

involved in the resistance selection dynamics, including the molecular mechanisms behind the resistance, the fitness cost and the location of the resistance gene on a mobile genetic element (Andersson *et al.* 2010).

Mutant selection window hypothesis

In the late 1990s, it was suggested that for each particular type of resistant mutant a dangerous antimicrobial concentration zone exists in which selective amplification can occur for that mutant type (Baquero *et al.* 1997a). Later on, Drlica and Zhao concluded that the sum of dangerous zones for all mutant types would represent a mutant selection window. This 'mutant selection window hypothesis' became one of the dominating theories on the selection of resistance. It states that selection of resistant mutants occurs in a concentration range spanning from the MIC of the susceptible strain to the MIC of the resistant mutant (Drlica 2003, Drlica *et al.* 2007). More specific, the lowest antimicrobial concentration that blocks the growth of the majority of the susceptible cells (99% inhibition = MIC_{99}) represents the lower boundary of the selection window. The Mutant Prevention Concentration (MPC) was designated as the upper limit of the selection window. The MPC is determined experimentally as the lowest concentration that allows no colony growth when more than 10^{10} cells are applied to antimicrobial-containing agar plates (Drlica 2003).

Hence, the mutant prevention window hypothesis predicts that mutant growth will be severely restricted if antimicrobial concentrations at the site of infection are maintained above the MPC throughout therapy. In addition, it predicts that resistance will arise rarely for antimicrobial drugs that have a narrow selection window, because in this case mutant amplification would occur only for short times.

Sub-MIC selection of resistance

The main focus of the mutant selection window hypothesis has been the MPC, and less attention was paid to concentrations below the MIC (sub-MIC concentrations). Later on, questions were raised about the possible impact of very low, residual concentrations of antimicrobials. Bacteria are frequently exposed to subinhibitory concentrations of antimicrobials, in the initial phase of therapy and during clearance of the antimicrobial from the body. Furthermore, due to excretion of unmetabolised antimicrobials in the urine or faeces, low concentrations of antimicrobials are found in the environment (Andersson *et al.* 2014, Finley *et al.* 2013). Cross-contamination of animal feed with antimicrobials, the use of antimicrobials as growth promotors (sub-MIC concentrations) and accidental under dosing are examples of routes by which subtherapeutic concentrations of antibiotics may modulate the intestinal microbiota in particular (Butaye *et al.* 2003, Filippitzi *et al.* 2016).

One of the first studies on low antimicrobial concentrations was published in the late 1990s (Baquero *et al.* 1997b). This study used *in vitro* models with mixtures of β-lactam susceptible and low-level resistant populations (*E. coli* and *Streptococcus pneumoniae*) and showed that four hours of antibiotic challenge (cefotaxime or amoxicillin) produced selective peaks of low-level resistant variant populations at low-level antibiotic concentrations. Later on, other studies followed that proved the potential of very low antimicrobial concentrations to select for resistant bacteria (Gullberg *et al.* 2014, Gullberg *et al.* 2011). These studies showed that resistance selection at sub-MIC levels is related to the bacterial fitness. As such, Gullberg and co-authors suggested an update on the selective window for resistance and introduced the Minimal Selective Concentration (MSC) (Gullberg *et al.* 2011). The MSC is defined as the lowest antimicro-

bial concentration that is needed to neutralize the fitness cost of each resistant determinant. The concentration range between the MSC and the MIC of the susceptible strain is referred to as the sub-MIC selective window (Gullberg *et al.* 2011). In **Figure 14**, the traditional selective window and sub-MIC selective window are presented graphically. The figure shows the MSC as the intercept of the growth curve of the susceptible strain and the growth curve of the resistant strain at increasing antimicrobial concentrations. In other words, as long as the growth rate of the susceptible strain is higher than the growth rate of the resistant strain, the susceptible strain will be able to outcompete the resistant strain (green interval). When the growth rate of the resistant strain exceeds the growth rate of the susceptible strain (that is, starting from the MSC), the resistant strain will be able to outcompete the susceptible strain.

It was found that the MSC of an antimicrobial can be much lower than the MIC. For example, the MSC of tetracycline in case of competition between a susceptible wild-type *Salmonella* Typhimurium strain and its isogenic resistant mutant appeared to be 100-fold lower than the MIC of the susceptible strain (Gullberg *et al.* 2011).

Hence, it is clear that resistant bacteria can be selected at sub-MIC concentrations, when the resistance mechanism confers a sufficiently high increase in resistance and concomitantly does not decrease fitness too much. It is suggested that the tendency will be that sub-MIC concentrations select faster for resistant bacteria with high fitness (regardless of resistance level), whereas concentrations above the MIC will select for mutants with high resistance (regardless of the fitness level) (Andersson *et al.* 2012).

Andersson and Hughes (2014) state that the selection of (mutational) antimicrobial resistance at sub-MIC levels differs in several important aspects from the selection at lethal antimicrobial concentrations. First, the

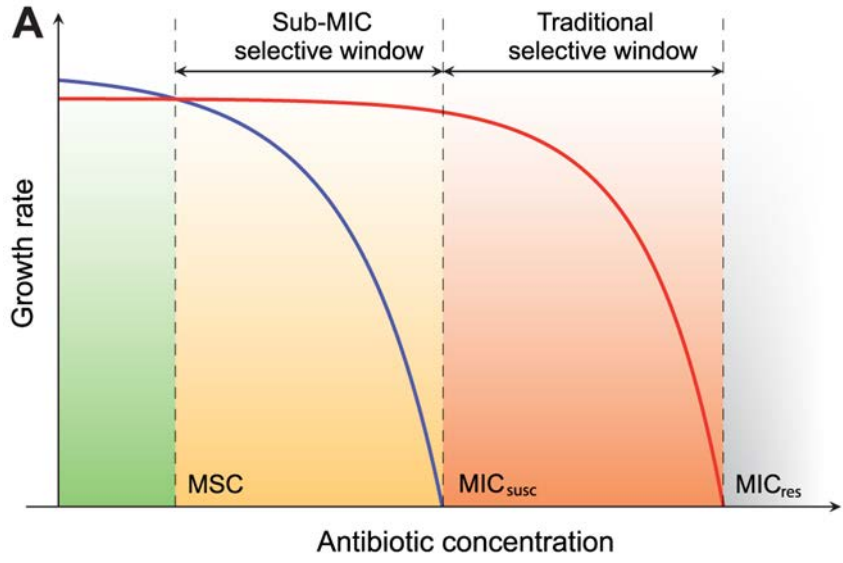


Figure 14. Growth rates as a function of antibiotic concentration.
Schematic representation of growth rates as a function of antibiotic concentration. Green indicates a concentration interval where the susceptible strain (blue line) will outcompete the resistant strain (red line). Orange (sub-MIC selective window) and red (traditional mutant selective window) indicate concentration intervals where the resistant strain will outcompete the susceptible strain. MIC_{susc} = minimal inhibitory concentration of the susceptible strain, MIC_{res} = minimal inhibitory concentration of the resistant strain and MSC = minimal selective concentration. Adopted from Gullberg *et al.* (2011).

'mutational space' is much greater at sub-MIC concentrations than at lethal concentrations of antimicrobials. At lethal concentrations, only the rare pre-existing mutants in a wild-type population that have high-level resistance will survive and the remaining susceptible population will be killed. In contrast, at sub-MIC concentrations most bacteria will grow slower but will not be killed, which will result in the emergence of a broader range of mutant variants. Second, it is suggested that selection under sub-MIC conditions tends to be progressive, meaning that it involves multiple

mutations that accumulate successively. As a consequence, it is also likely that sub-MIC conditions select for 'mutator bacteria'. This kind of bacteria show increased mutation rates, typically as a result of inactivating mutations in DNA repair mechanisms, and can thus adapt quickly to a changing environment (Mao *et al.* 1997). Third, sub-MIC antimicrobial concentrations might also affect the rate of horizontal gene transfer, recombination and mutagenesis. At lethal antimicrobial concentrations, these effects are less important, as the susceptible cells are killed before any of the associated phenotypes can be expressed or before genes can be transferred. And fourth, the association between low fitness cost and sub-MIC resistance selection could have an important implication. It means that restriction of antimicrobial use as a means to counter-select resistant populations could be less effective in populations in which resistance has emerged under sub-MIC conditions (Andersson *et al.* 2010, Andersson *et al.* 1999). However, more research is needed to confirm the latter hypothesis.

4.4.2 HORIZONTAL GENE TRANSFER

Introduction

Selection of resistant bacteria is a major contributing factor to the spread of antimicrobial resistance, but also transfer of resistance genes plays a key role in this phenomenon. This spread of resistance also occurs without the selective pressure of antimicrobials (Smet *et al.* 2011).

For most bacteria, the genetic material comprises one circular chromosome, which always carries the housekeeping genes. They may carry additional DNA as plasmids. Sexual reproduction does not occur in bacteria, they divide asexually through binary fission. 'Vertical gene transfer' refers to the transfer of genetic material from a parent to the offspring. Besides that, an important share of genetic material exchange happens through

'horizontal gene transfer' between (possibly non-related) bacterial cells. This can be achieved by means of three main mechanisms: transformation (**Figure 15a**), transduction (**Figure 15b**) and conjugation (**Figure 15c**). When it comes to transfer of antimicrobial resistance genes, conjugation is considered as the most important mechanism because many resistance genes are associated with conjugative or mobilizable elements as transposons and plasmids (Boerlin *et al.* 2008, Hawkey *et al.* 2009). In addition, conjugation events are not limited to bacteria of the same species, but can also occur between species of different species or genera (Mathur *et al.* 2005).

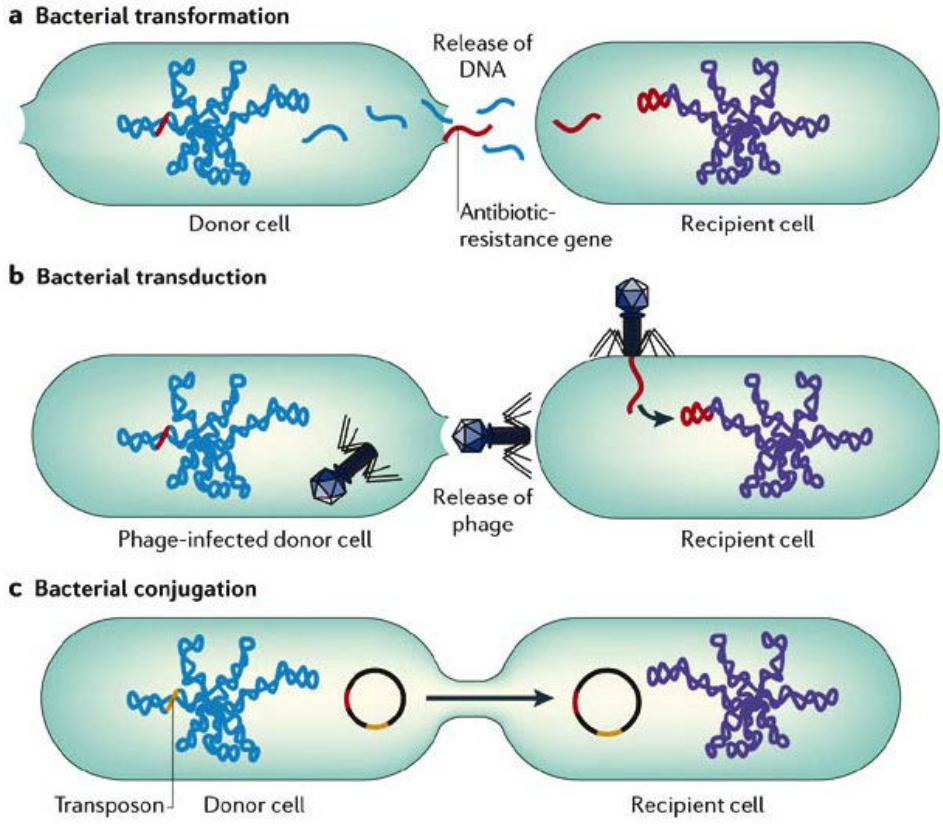


Figure 15. Mechanisms of horizontal gene transfer. Adopted from Furuya *et al.* 2006.

Mechanisms

Conjugation

Transfer of DNA through conjugation (**Figure 15c**) involves a multi-step process that requires cell to cell contact between a donor and recipient cell, which is achieved via cell surface pili, named sex pili. The conjugative machinery is encoded by a set of genes that can be located on autonomously replicating plasmids ('conjugative plasmids') or on the chromosome (e.g. 'integrative conjugative elements'). These genes may also enable the mobilization of non-conjugative plasmids ('mobilizable plasmids') and integrative and mobilizable elements (Smillie *et al.* 2010).

The mechanisms to achieve cell-cell contact differ between Gram negative and Gram positive bacteria. In Gram negative bacteria, this first step in conjugation is promoted by extracellular filaments called sex pili. These form a mating bridge that serves as a conduit for DNA. Other mechanisms, such as pheromones secreted by the recipient cells or aggregation, induce the cell-cell contact between Gram positive bacteria (Grohmann *et al.* 2003, Smillie *et al.* 2010). The second step in the conjugation process is the transfer of a single stranded linear DNA molecule from the donor to the recipient cell. The incoming plasmid is then established in the recipient cell by relaxase-mediated circularization and synthesis of the complementary strand.

Plasmids (**Figure 16**) are classified in 'Inc groups' according to their incompatibility. Incompatibility is defined as the inability of two related plasmids to be propagated stably in the same cell line; thus, only compatible plasmids can be rescued in transconjugants (Carattoli 2009).

Antimicrobial resistance genes are in many cases associated with conjugative plasmids and transfer of these plasmids has been observed in many types of ecosystems such as soils, water, food, health care settings,

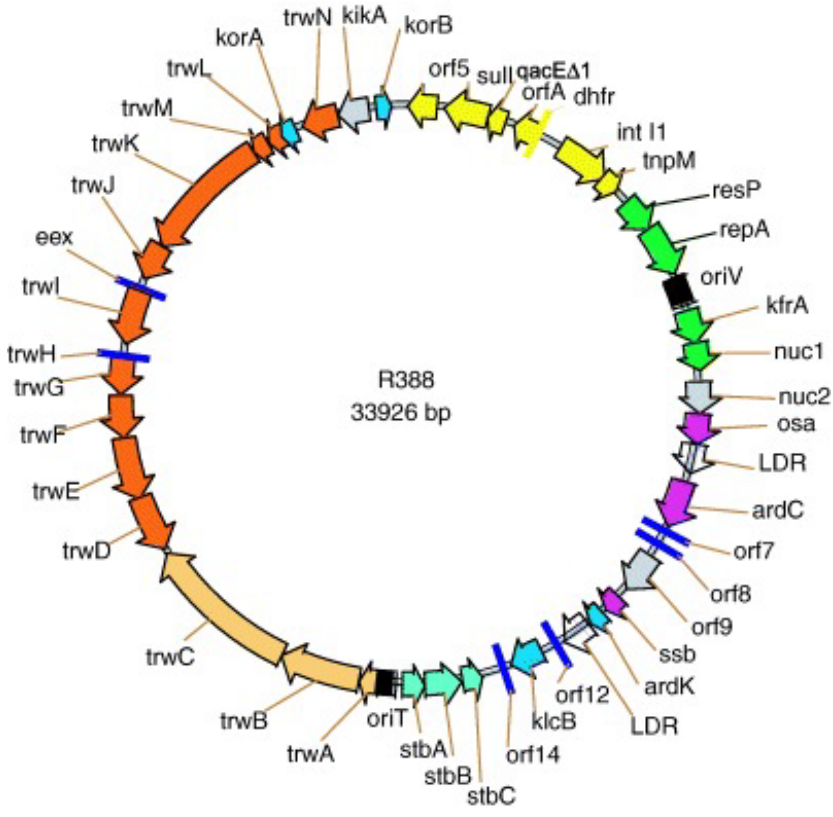


Figure 16. Example of a conjugative plasmid. This plasmid carries genes encoding for conjugation (orange), an integron (yellow) with antimicrobials resistance gene cassettes, transfer and replication origins (black), etc. Adopted from Fernandez-Lopez *et al.,* 2006.

the intestines,… (Davison 1999). Hence, plasmids, together with transposons, are considered as the most important mobile genetic element in the dissemination of resistance genes.

Transposons (Tn) are genetic elements with the ability to move from one genetic location to another (**Figure 17**). This is accomplished via an enzyme called transposase, and the gene encoding this protein is generally located on the transposon itself. Due to the transposase, transposons can insert randomly, independently of sequence homology.

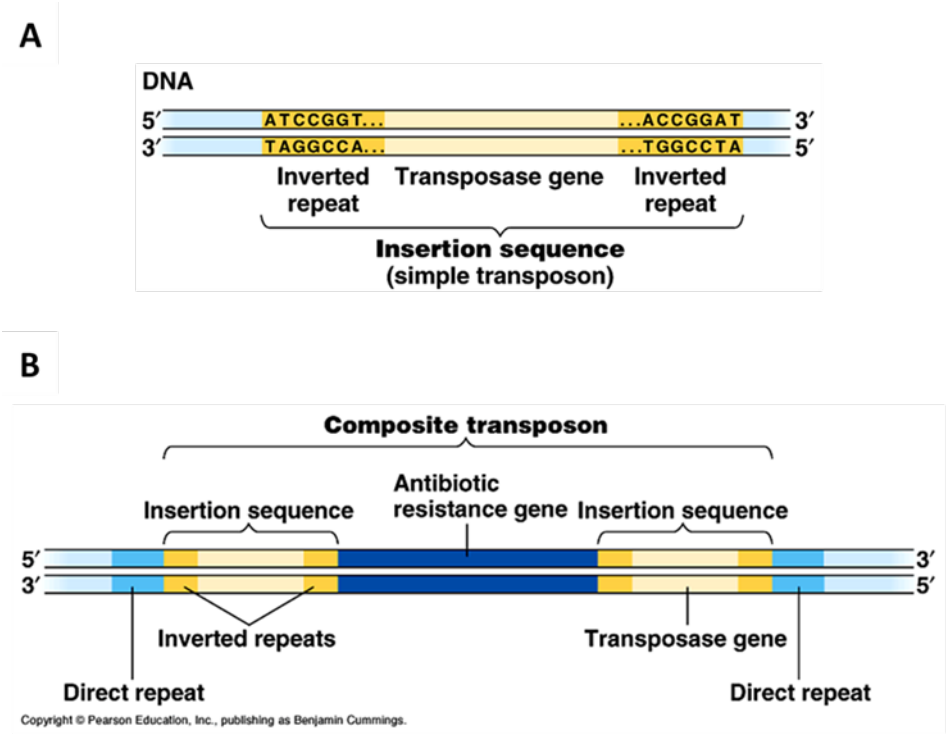


Figure 17. (A) **Insertion sequences**, the simplest transposable elements in bacteria, contain a single gene that encodes transposase, which catalyzes movement within the genome. The inverted repeats are backward, upside down version of each other; only a portion is shown. The inverted repeat sequence varies from one insertion sequence type to another. **(B) Transposons** contain one or more genes in addition to the transposase gene. In the transposon shown here, an antimicrobial resistance gene is located between twin insertion sequences. The resistance gene is carried along as part of the transposon when the transposon is inserted at a new site in the genome.

In analogy with plasmids, transposons can be subdivided into two types: conjugative and mobilizable transposons. The conjugative transposons are part of a larger group of mobile genetic elements, the integrative and conjugative elements (ICEs). These elements have the ability to integrate into and excise from the chromosome, replicate within the chromosome and are transferred by conjugation (Burrus *et al.* 2002). Mobilizable transposons belong to the group of integrative mobilizable elements (IMEs). The

smallest type of transposons, consisting only of the transposase gene and the flanked inverted repeats, are also called **insertion sequences** (**Figure 17**). They are very common and can be found in almost all bacteria and on many conjugative plasmids (Siguier *et al.* 2006). A specific type of insertion sequences, the insertion sequence common regions elements (IS*CR*) are particular of interest because of their close association with a wide variety of antimicrobial resistance genes and their association with integrons (Toleman *et al.* 2006).

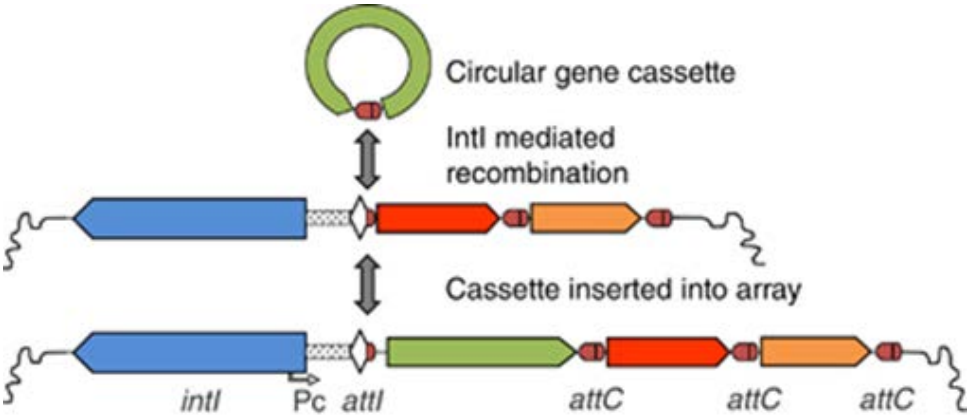


Figure 18. Integron structure and function. Integrons consist of a gene for an integron-integrase (*intI* in case of this class 1 integron) that catalyses recombination between the *attC* site of circular gene cassettes and the attendant integron recombination site, *attI*. This activity results in the sequential insertion of multiple, different cassettes to form a tandem cassette array that, in some cases, might contain hundreds of different genes. Inserted genes are expressed by an integron-encoded promoter, Pc. Adopted from Gillings *et al.* (2015).

Integrons (**Figure 18**) are recombination based genetic systems that use modular genetic cassettes to generate genomic diversity (Gillings 2014). These immobile elements consist of three basic components: two 'conserved regions' (5'-CS and 3'-CS) and between these regions a variable

number of gene cassettes. Variations on this basic structure have been reported, such as integrons with a deletion of the 3'-CS region (Hall *et al.* 1994) and the so called complex integrons (Quiroga *et al.* 2007, Sabaté *et al.* 2002). All the elements necessary for the integration, expression and excision of gene cassettes are located within the 5'-CS region, namely an *intI* gene (encoding a site-specific recombinase), a common promotor (Pc) and a recombination site *attI*. A gene cassette consists of a gene and a recombination site attC, by which the cassette can be integrated in the integron by site-specific recombination (Cambray *et al.* 2010, Gillings 2014). When integrons are associated with mobile genetic elements such as plasmids or transposons, they are called mobile integrons. They can also be located on the bacterial chromosome (chromosomal integrons), but antimicrobial resistance gene cassettes are mostly integrated in mobile integrons (Domingues *et al.* 2012). Currently, five different classes of mobile integrons have been described, although until now only three of them have been associated historically with dissemination of resistance (Cambray *et al.* 2010). The class 1 integrons are more prevalent than other integron classes and are the most ubiquitous class of integrons in Enterobacteriaceae (Kaushik *et al.* 2017). Today, bacteria carrying multidrug resistance integrons are widespread, both in humans, animals and the environment. A Spanish study showed that up to 80% of commensal *E. coli* isolates from farm animals can carry integrons (Marchant *et al.* 2013).

Transduction

Transfer of DNA between bacteria that is enabled by bacterial viruses (bacteriophages), is called transduction (**Figure 15b**). When bacteriophages infect and lyse bacteria, bacterial host DNA can be integrated in the phage genome or accidentally be packed instead of the viral genome. When they

subsequently infect a new host, these bacterial genes can be transferred and integrated into the genome of the recipient cell (Muniesa *et al.* 2013).

In the past, the role of transduction in the dissemination of antimicrobial resistance has received less attention than conjugation and transformation, but now the contribution of transduction in horizontal gene transfer is estimated to be larger than first perceived (Balcazar 2014, Muniesa *et al.* 2013). Especially in livestock manure, hospital effluent water and sewage and river water, high numbers of phage particles carrying resistance genes have been found (Colomer-Lluch *et al.* 2011, Marti *et al.* 2014). Moreover, it has been shown that the fraction of phages carrying antimicrobial resistance genes increase drastically in the gut of mice treated orally with ampicillin or ciprofloxacin (Modi *et al.* 2013).

Transformation

Natural genetic transformation is defined as the active uptake of free DNA by bacterial cells and the heritable incorporation in the genome (Lorenz *et al.* 1994). It involves several steps: the release of DNA from cells, uptake of the free DNA by competent cells, stable integration of the DNA in the recipient cell and finally the expression of the acquired trait. In other words, several conditions have to be met in order for transformation to take place (Thomas *et al.* 2005). Bacteria can release DNA passively after cell death or actively by secretion (Nielsen *et al.* 2007). Several bacterial species belonging to different phyla possess natural competence for transformation, whereas others are able to undergo transformation under adequate conditions (Johnston *et al.* 2014). Natural competence is defined as a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA (Dubnau 1999). In addition, competence can be induced under laboratory conditions in a wide range of bacteria (Aune *et al.* 2010).

Importantly, different studies have shown that competence for transformation can be induced in many species of bacteria by exposure to antimicrobials (Charpentier *et al.* 2011, Charpentier *et al.* 2012, Prudhomme *et al.* 2006). This could mean that antimicrobial exposure could stimulate transformation of antimicrobial resistance genes.

Mobility of tetracycline resistance genes

Since 1953, tetracycline-resistant bacteria have been found increasingly in humans, animals, food and the environment. Tetracycline resistance is usually the result of the acquisition of new genes and is primarily due to either energy-dependent efflux of tetracycline or protection of the ribosomes from its action. The first reports on tetracycline resistance determinants were made decades ago in Japan (Roberts 1996). The so called *tet* genes are often associated with plasmids, transposons and conjugative transposons, which can also carry other resistance determinants and/or heavy metal resistance genes. The type of element a specific *tet* gene is associated with, may greatly influence its ability to spread to new bacterial genera (Chopra *et al.* 2001). So far, *tet* genes have not been found as gene cassettes within integrons. Gram negative efflux genes are often associated with conjugative plasmids, whereas Gram positive efflux genes are mostly found on small mobilizable plasmids or in the chromosome associated with a transposon. These transposons have a wide host range covering both Gram negative and Gram positive bacteria. Likewise the ribosomal protection proteins are widely distributed among the different genera (Roberts 1996).

Well studied *tet* genes, such as *tet*(B), *tet*(M) or *tet*(A) (often located on transposons Tn10, Tn916 and Tn1721 respectively), have been shown to be widely distributed (Allmeier *et al.* 1992, Roberts 1996). This supports the hypothesis that *tet* genes are exchanged by bacteria from many different

ecosystems. This means that bacteria exposed to antimicrobials in the environment or in animals can ultimately influence antimicrobial resistance in bacteria of both animal and human origin.

Horizontal gene transfer at sub-MIC antimicrobial levels

It is generally assumed that antimicrobials promote horizontal gene transfer, even at sub-MIC level (Aminov 2011, Andersson *et al.* 2014, Beaber *et al.* 2004). In the 1980s, it was already reported that sub-MIC concentrations of β-lactams promoted the transfer of tetracycline resistance plasmids by up to 1,000-fold in *Staphylococcus aureus* (Barr *et al.* 1986). Another study showed that sub-MIC tetracycline concentrations accelerated the mobilization of a resident non-conjugative plasmid by chromosomally encoded tetracycline conjugal elements in *Bacteroides* sp. (Valentine *et al.* 1988). Later on, it was discovered that similar exposure conditions also enhanced the excision and conjugal transfer of the ICE CTnDOT (Stevens *et al.* 1993, Whittle *et al.* 2002). Other mobile genetic elements of which transfer has been shown to be stimulated by sub-MIC tetracycline concentrations are the ICEs *Tn*916 and *Tn*925, both generally carrying the *tet*M gene (Showsh *et al.* 1992, Torres *et al.* 1991). These (and other) pioneer studies were conducted *in vitro*, using standard mating techniques. The next step was to verify whether similar effects would arise in *in vivo* situations. Experiments with gnotobiotic lab animals confirmed that the stimulatory effect of sub-inhibitory antimicrobials on transfer of mobile genetic elements also occurs in real *in vivo* situations. However, the intestinal microbiota of gnotobiotic animals still lack the high diversity and density of the microbiota of normal animals.

Different mechanisms are responsible for the enhanced movement of mobile genetic elements in the presence of antimicrobials. At sublethal

concentrations, antimicrobials can increase the conjugation rate by activating the excision of transferrable genes from the host chromosome and/or by inducing the expression of the conjugation machinery, e.g. in the case of conjugative transposon CTnDOT (Jeters *et al.* 2009, Moon *et al.* 2005, Whittle *et al.* 2002). It has also been speculated that antimicrobials can induce general cellular responses, such as upregulation of key survival genes (Zhang *et al.* 2013) and induction of the SOS response (Beaber *et al.* 2004, Zhang *et al.* 2000) which could indirectly promote conjugation (Andersson *et al.* 2014). This kind of event has been reported e.g. for *Vibrio cholerae* following ciprofloxacin exposure, for *Staphylococcus aureus* following β-lactam exposure and for *E. coli* following the exposure to the combination of kanamycin and streptomycin (Beaber *et al.* 2004, Maiques *et al.* 2006, Zhang *et al.* 2013). Furthermore, the integron integrase genes are often activated in the cell via the SOS response, suggesting that antibiotic treatment can increase the activity of integrons in bacteria (Guerin *et al.* 2009).

Interestingly, also the opposite effect, i.e. the inhibition of conjugation, has been reported e.g. in case of exposure of *E. coli* to quinolones (Viljanen *et al.* 1991, Weisser *et al.* 1987).

A variety of confounding factors can however complicate the interpretation of conjugation studies, which makes that the mechanisms by which antimicrobials modulate horizontal gene transfer remain today poorly understood. It is still unclear whether antimicrobials regulate the efficiency of horizontal gene transfer directly, or serve as a selection force to modulate population dynamics after such gene transfer has occurred, or both (Lopatkin *et al.* 2016). Lopatkin and co-authors decoupled antimicrobial mediated induction of the conjugation machinery from the global effect an antimicrobial might have on the conjugation efficiency and the conjugation event from the ensuing growth dynamics. Thus, it was shown that

particularly the physiological state of cells before conjugation and energy availability during conjugation, increases the conjugation efficiency substantially. These findings underline the importance of quantifying the growth dynamics of microbial populations to estimate both the physiological state of cells and the effects of antimicrobial mediated selection (Lopatkin *et al.* 2016).

4.4.3 SUB-MIC RESISTANCE SELECTION AND TRANSFER IN THE PORCINE INTESTINAL MICROBIOTA

The majority of the mammalian gut microbiota is represented by *Bacteria,* while *Archaea*, *Protozoa*, viruses, and fungi are present to a lesser extent (Leser *et al.* 2009, Sommer *et al.* 2013). The porcine gut microbiota comprises relatively few different phyla, i.e. the *Bacteroidetes* and *Firmicutes.* The most abundant genera are *Prevotella, Lactobacillus, Treponema, Roseburia,* and *Streptococcus,* as in many other animal species (Holman *et al.* 2014, Kim *et al.* 2011, Lamendella *et al.* 2011, Park *et al.* 2014). The intestinal microbiota is not homogenous: it depends on the individual, the sample location and age (Holman *et al.* 2014, Isaacson *et al.* 2012, Leser *et al.* 2000, Mach *et al.* 2015).

The currently available studies in literature concerning selective pressure of sub-MIC concentrations of antimicrobials in the porcine microbiota, mostly consider growth promoting agents such as chlortetracycline and tylosine. Early studies on this topic mostly investigated the cultivable porcine microbiota. For example, Dawson and co-authors described the effect of therapeutic (220 mg/kg feed) and subtherapeutic (27.5 mg/kg feed) dosages of chlortetracycline on the total cultivable anaerobic population (Dawson *et al.* 1984). They only found significant higher presence of resistant anaerobes in the group that received the therapeutic dose. A more recent study evaluated antimicrobial resistance in total anaerobes isolated

from the faeces of pigs fed either tylosin or virginiamycin and recorded that all anaerobes were resistant to both antibiotics, even in the absence of exposure (Kalmokoff *et al.* 2011). Another study reported a rapid significant increase in tylosin-resistant faecal anaerobes in pigs that were fed tylosin-supplemented feed (Holman *et al.* 2013). On the other hand, the subtherapeutic dose of chlortetracycline (5.5 mg/kg feed) had no measurable effect on chlortetracycline resistance in total anaerobes (Holman *et al.* 2013).

The challenges associated with culturing gut-associated microorganisms together with the progress in molecular techniques have led to the use of culture-independent methods such as qPCR, single gene sequencing and metagenomic analysis to investigate the (porcine) microbiota. Several recently published studies describe the effects of subtherapeutic antimicrobial concentrations on the porcine microbiota using these methods. However, each of these studies lacks information on intestinal antimicrobial concentrations resulting from the in-feed administration. Rettedal and coauthors reported that in piglets that were fed chlortetracycline for two weeks at a subtherapeutic dose (50 mg/kg feed) following weaning, a significant change in the ileal microbiota was observed. These changes were mainly associated with a decrease in *Lactobacillus johnsonii*, *Turicibacter*, and an increase in *Lactobacillus amylovorus* (Rettedal *et al.* 2009). Another study investigated the effect of in-feed ASP250 [i.e. a combination of the performance enhancing antimicrobials chlortetracycline (100 mg/kg feed), sulphamethazine (100 mg/kg), and penicillin (50 mg/kg)] administered to three post-weaned pigs for three weeks on the abundance and diversity of antimicrobial resistance genes. They reported an increase in the diversity of antimicrobial resistance genes as well as an increase in the abundance of six different classes of resistance genes in the medicated pigs. These resistance gene classes included not only those which confer resistance to the

administered antimicrobials but also to antimicrobials not used in the study, which points to potential co-selection of these resistance gene classes (Looft *et al.* 2012). Furthermore, ASP250 caused shifts in the porcine microbiota, among which a decrease in the relative abundance of *Bacteroidetes* and an increase in *Proteobacteria*, of which most were *E. coli* (Looft *et al.* 2012). Interestingly, many of these culture-independent studies demonstrate that there is a wide dissemination of antimicrobial resistance determinants among pigs in modern production facilities, even in the absence of antimicrobial exposure (Agga *et al.* 2015, Holman *et al.* 2013, Kalmokoff *et al.* 2011, Pakpour *et al.* 2012). Regarding tetracycline resistance, in particular *tet*(M), *tet*(O), *tet*(Q), and *tet*(X) were frequently detected in pigs that were not directly exposed to antimicrobials (Agga *et al.* 2015, Barkovskii *et al.* 2012, Holman *et al.* 2013).

5 CLOSING REMARKS

Antimicrobial feed medication is frequently applied in the pig industry. An unavoidable consequence of this practice is the unintended cross-contamination of feed with low concentrations of antimicrobials due to carry-over of antimicrobials from medicated feed to non-target feed. There is no European regulation that lays down maximum levels of antimicrobial carry-over valid yet, but a proposal for a regulation on the manufacture, placing on the market and use of medicated feed (repealing Council Directive 90/167/EEC) is currently debated.

Based on the literature discussed above, it is to be expected that antimicrobial concentrations in the intestinal microbiota due to cross-contaminated feed may select for resistance. The microbiota in the large intestines are particularly of interest, as they harbour the highest microbial diversity in the intestinal tract.

However, it has not been investigated if these low antimicrobial concentrations in the feed reach the pig's large intestines and if so, whether these concentrations influence resistance selection.

CHAPTER 2

General aims

Antimicrobial resistance is one of the most important 'One Health' issues. It has been a hot topic in research since years, and thus numerous studies on resistance mechanisms, selection, and transfer have been published. Nevertheless, antimicrobial resistance is a very complex phenomenon and still many knowledge gaps are left to fill. One of these gaps concerns the effects of low antimicrobial concentrations, such as carry-over concentrations in pig feed, on resistance selection.

The general aim of this doctoral thesis was therefore to evaluate the effect of 3% carry-over concentrations of antimicrobials in pig feed on resistance selection and transfer in the porcine intestinal microbiota. The choice for 3% carry-over was based on the only legal rule on maximum allowed carry-levels valid at that time, namely the European legislation on coccidiostats and histomonostats in animal feed (EP 2003).

This main goal was translated into three research questions:

— Which antimicrobial concentrations can be found in the gut of pigs that are fed with feed that contains carry-over levels of 3% of the recommended dose of chlortetracycline, doxycycline and sulphadiazine-trimethoprim? This question is answered in **Chapter 3**.
— What are the effects of intestinal doxycycline concentrations caused by 1% and 3% carry-over levels in the feed on the selection of doxycycline resistant porcine commensal *E. coli* strains and on the transfer of their resistance plasmids in an *in vitro* model with pure cultures? This question is answered in **Chapter 4.**
— What are the effects of intestinal doxycycline concentrations caused by 1% and 3% carry-over levels in the feed on the selection of a doxycycline resistant porcine commensal *E. coli* strain and on the transfer of its resistance plasmid in an *ex vivo* model simulating the microbiota of the pig caecum? This question is answered in **Chapter 5**.

CHAPTER 3

Residues of chlortetracycline, doxycycline and sulphadiazine-trimethoprim in intestinal content and faeces of pigs due to cross-contamination of feed

Adapted from:

Peeters, L. E., Daeseleire, E., Devreese, M., Rasschaert, G., Smet, A., Dewulf, J., Heyndrickx, M., Imberechts, H., Haesebrouck, F., Butaye, P. and Croubels, S. (2016). Residues of chlortetracycline, doxycycline and sulphadiazine-trimethoprim in intestinal content and faeces of pigs due to cross-contamination of feed. *BMC Veterinary Research*, 12: 209.

1 ABSTRACT

Background: Cross-contamination of feed with low concentrations of antimicrobials can occur at production, transport and/or farm level. Concerns are rising about possible effects of this contaminated feed on resistance selection in the intestinal microbiota. Therefore, an experiment with pigs was set up, in which intestinal and faecal concentrations of chlortetracycline (CTC), doxycycline (DOX) and sulphadiazine-trimethoprim (SDZ-TRIM) were determined after administration of feed containing a 3% carry-over level of these antimicrobials.

Results: The poor oral bioavailability of tetracyclines resulted in rather high concentrations in caecal and colonic content and faeces at steady-state conditions. A mean concentration of 10 mg/kg CTC and 4 mg/kg DOX in the faeces was reached, which is higher than concentrations that were shown to cause resistance selection. On the other hand, lower mean levels of SDZ (0.7 mg/kg) and TRIM (< limit of detection of 0.016 mg/kg) were found in the faeces, corresponding with the high oral bioavailability of SDZ and TRIM in pigs.

Conclusions: The relation between the oral bioavailability and intestinal concentrations of the tested antimicrobials, may be of help in assessing the risks of cross-contaminated feed. However, future research is needed to confirm our results and to evaluate the effects of these detected concentrations on resistance selection in the intestinal microbiota of pigs.

2 INTRODUCTION

Group administration of veterinary drugs through feed and drinking water is frequently applied in the pig industry. Antimicrobials are often administered to pigs by mixing the feed with an oral powder or premix formulation (BelvetSAC 2015, Callens *et al.* 2012, EMA 2015). The important role of group administration of antimicrobials in the selection of resistant bacteria is generally recognized (FASFC 2013). Concerns about antimicrobial resistance selection have already led to the prohibition of use of antimicrobials as growth promoters in Europe since 2006 (EP 2003). However, group medication is still used extensively in many countries for prophylactic, metaphylactic and therapeutic purposes (Callens *et al.* 2012). Major disadvantages of group medication are the poor control over dosage due to differences in feed uptake between sick and healthy animals, inflexible therapy duration for medicated feed, the risk of carry-over and the inevitable contamination of the environment with antimicrobials (FASFC 2013). Different types of antimicrobial formulations can be used to treat animals in group. Premixes (38.2%), oral powders (33.7%) and solutions (19.6%) each accounted for a significant share of the total amount of sold antimicrobials in 26 European countries in 2013 (EMA 2015). However, the types of antimicrobial formulations used for group treatment vary considerably between the individual countries. In some countries, such as Germany, Luxembourg, Estonia and Denmark, it seems that oral powders and solutions are preferred over premix formulations, whereas the opposite applies for countries like Spain, Portugal, Hungary, Cyprus and the UK (EMA 2015). In Belgium, both oral powders (70%, for feed and drinking water) and premixes (20%) are used frequently (BelvetSAC 2015, EMA 2015). In this study we focus on medicated feed produced in feed mills, and thus on premix formulations.

Carry-over of feed additives and veterinary drugs from a compound feed to a non-target feed is a problem inherent to the production of compound feed in feed mills and the transport, storage and delivery of these feeds (Borras *et al.* 2011, Stolker *et al.* 2013). A batch of non-target feed that is produced directly after a compound feed, is generally called 'flushing feed'. So far, only coccidiostats and histomonostats are included in the European legislation regarding maximum allowed levels (3%) in flushing feeds (EP 2002). In Belgium, a covenant was established in 2013 between the Belgian Federal Agency for the Safety of the Food Chain (FASFC) and the Belgian Feed Association (BFA) (BFA and FASFC 2013), in which guidelines for maximum levels of carry-over were set for antimicrobials (1% of the min. approved dose, except 2.5% for some formulations in rabbit pellet feed), paracetamol (1%) and anthelmintics (1-3% of the max. approved dose, depending on the type of feed). Moreover, due to additional technical requirements for feed mills established in this covenant, namely adding the drugs or additives at the end of the production line instead of the middle, carry-over should be reduced significantly in Belgian feed mills. Unfortunately, carry-over between different feed batches occurs not only in feed mills, but also during transport and at farm level, which makes it a difficult issue to control (McEvoy 2002). A study by Putier et al. (2010), investigating carry-over at transport level, indicated that this route should not be underestimated. In this study, two types of carry-over (inter-bin and intra-bin) of antimicrobials (oxytetracycline and chlortetracycline) were measured in ten different types of delivery trucks. Inter-bin carry-over of the two antimicrobials ranged from 0.04 to 1.41% and intra-bin carry-over ranged from 0 to 0.44%. Carry-over at farm level remains to be elucidated but could be of great importance, especially in countries with a focus on use of oral powders and solutions as these products are mixed with feed or water at the farm (EMA 2015).

As a result of cross-contamination of feed, the intestinal microbiota of pigs can be exposed to unintended, low concentrations of antimicrobials (Zuidema *et al.* 2014). It is known that low antimicrobial concentrations can evoke selection of resistant bacteria *in vitro* (Gullberg *et al.* 2014, Gullberg *et al.* 2011) and *in vivo* (Brewer *et al.* 2013). Moreover, *in vitro* studies with tetracycline, trimethoprim, streptomycin, erythromycin and ciprofloxacin show that the fitness cost for resistance-conferring mutations or genes selected at sub-MIC concentrations is often lower than for those selected above the MIC (Gullberg *et al.* 2014, Gullberg *et al.* 2011). Therefore, these sub-MIC selected mutants would be more stable in bacterial populations and thus potentially more problematic than mutants selected above the MIC (Andersson *et al.* 2010, Liu *et al.* 2011, Sandegren 2014).

In order to assess the true effect of cross-contaminated feed on resistance selection in the intestinal microbiota, it is necessary to first determine the intestinal concentrations of antimicrobials after administration of such feed. Indeed, each type of antimicrobial has different PK properties that determine the fraction of the orally ingested antimicrobial that remains in the intestines or is excreted in the bile (Olofsson *et al.* 2007). The oral bioavailability is a measure of the rate and extent of a drug reaching the systemic circulation in its unchanged form through intestinal absorption (Hu *et al.* 2011). As such, this PK property has a significant impact on the fraction of drug that remains in the intestinal content. The oral bioavailability is strongly dependent on the active substance and may be influenced among others by the formulation type and prandial state of the animal. In this study, an *in vivo* experiment with pigs was set up to determine concentrations in the intestinal content and the faeces of chlortetracycline (CTC), doxycycline (DOX) and sulphadiazine-trimethoprim (SDZ-

TRIM) when administering feed that contains 3% of the maximum recommended dose. This percentage was chosen considering the only legally applicable guideline in Belgium regarding maximum carry-over levels at the time of the experiment (2013) (EP 2002). The choice of antimicrobials was based on two aspects. First, tetracyclines and sulphonamides are among the most used classes of antimicrobials in Belgium when considering oral administration (2013). Second, the oral bioavailability in pigs was taken into account. SDZ, typically used in a combined formulation with TRIM because of the synergistic mode of action, has a very high oral bioavailability in pigs, namely 85-100% (Baert *et al.* 2001, Nielsen *et al.* 1994). The same applies to TRIM (73-92%) (Baert *et al.* 2001, Nielsen *et al.* 1994). In contrast, tetracyclines have a low oral bioavailability in pigs, with CTC even lower (6%) than DOX (21-50%) (Baert *et al.* 2000, Nielsen *et al.* 1996, Sanders *et al.* 1996).

In the past, studies have been performed to examine levels of antimicrobials and other drugs in tissues and eggs when poultry is fed with cross-contaminated feed (Segato *et al.* 2011, Vandenberge *et al.* 2012a, Vandenberge *et al.* 2012b). Yet no data have been published regarding intestinal concentrations due to cross-contamination in pigs or other livestock. The aim of this study was therefore to determine intestinal concentrations in pigs of CTC, DOX and SDZ-TRIM, when they were fed a diet that contains a 3% carry-over level of these antimicrobials.

3 MATERIALS AND METHODS

3.1 PREMIXES, REAGENTS AND STANDARDS

The premixes used for the preparation of the experimental diets were Doxyprex® (active pharmaceutical ingredient, API: 100 mg DOX hyclate/g premix), provided by Kela Veterinaria (Sint-Niklaas, Belgium), Aurofac® (API: 250 mg CTC.HCl/g premix) and Tucoprim® (API: 125 mg SDZ/g premix and 25 mg TRIM/g premix), both provided by Zoetis (Brussels, Belgium). Analytical standards of DOX (doxycycline hyclate), CTC (chlortetracycline.HCl), SDZ and TRIM were obtained from Sigma-Aldrich (Bornem, Belgium). The internal standards (IS) were demethylchlortetracycline.HCl (DMCTC, Sigma-Aldrich) and $^{13}C_6$-sulphadimethoxine and d9-trimethoprim, both from Witega (Berlin, Germany). Methanol (CH_3OH) and acetonitrile (CH_3CN) were of LC-MS (liquid chromatography – mass spectrometry) grade and obtained from Biosolve (Valkenswaard, The Netherlands). Water was of LC-MS grade and was obtained from Biosolve (Valkenswaard, The Netherlands) for tetracycline analysis, and was generated from a Milli Q gradient purification system (Millipore, Billerica, MA, U.S.) for SDZ and TRIM analysis. Acetic acid (CH_3COOH, >99.99%) was from Sigma Aldrich, succinic acid ($C_4H_6O_4$) from VWR (Leuven, Belgium) and sodium sulphate (Na_2SO_4), formic acid (HCOOH), trichloroacetic acid (CCl_3COOH) and sodium hydroxide (NaOH) were from Merck (Darmstadt, Germany).

3.2 PREPARATION OF STANDARD SOLUTIONS

Standard stock solutions of CTC, DOX and the IS DMCTC were prepared in CH_3OH at a concentration of 1 mg/ml and stored at ≤ -15 °C. Working solutions of DMCTC at a concentration of 100 µg/ml and 20 µg/ml, and of

CTC and DOX at a concentration of 100 µg/ml were prepared by appropriate dilution with water. Standard stock solutions of SDZ and the IS $^{13}C_6$-sulphadimethoxine were prepared in CH_3CN/water (50/50, V/V) at a concentration of 1 mg/ml and stored at ≤ -15 °C. Standard stock solutions of TRIM and the IS d9-trimethoprim were prepared in CH_3OH and stored at ≤ -15 °C. For $^{13}C_6$-sulphadimethoxine and d9-trimethoprim, working solutions of 1 µg/ml were prepared in water making use of an intermediate working solution of 10 µg/ml in CH_3CN/water (50/50, V/V). SDZ and TRIM working solutions of 10 µg/ml, 1 µg/ml and 0.1 µg/ml were prepared in water and used for spiking the calibration samples. To prepare sodium succinate 0.1 M, 11.8 g of $C_4H_6O_4$ was dissolved in 600 ml of water, 10 M NaOH was added until pH 4.0 was reached, and water was added to obtain a final volume of 1000.0 ml. The solution was stored at 4.0 °C. Solutions of HCOOH (0.1%), CCl_3COOH (20%) and CH_3COOH (0.1%) were prepared by appropriate dilutions with water.

3.3 PREPARATION OF THE EXPERIMENTAL FEED

Three different batches of experimental diets were prepared. Blank feed (meal n° 9231, AVEVE, Merksem, Belgium) was mixed with the DOX, CTC and SDZ-TRIM premixes, respectively. A custom made mixing device (Silobouw, Zulte, Belgium) was kindly provided by the Food Science and Technology Unit of Ghent University. The added amounts of antimicrobials were calculated to yield cross-contamination levels in the feed corresponding to 3% of the maximum recommended dose, MRD (CTC, 18.6 mg/kg BW (body weight)/day; DOX, 13.5 mg/kg BW/day; SDZ, 25.0 mg/kg BW/day; TRIM, 5.0 mg/kg BW/day). BW and daily feed intake were set at 25 kg and 1.5 kg respectively. Thus, a target concentration of 9.29 mg CTC/kg feed, 6.76 mg DOX/kg feed, 12.5 mg SDZ/kg feed and 2.50 mg TRIM/kg feed was aimed for. Each premix was first mixed manually with

10 kg of blank feed, which was then mixed with the remaining blank feed (120 kg) in the feed mixer for 25 min. The feed was collected from the mixer in 13 bags, each containing 10 kg. One sample of approximately 200 g was taken from bag n° 1, 2, 3, 5, 6, 8, 9, 11, 12 and 13 of each experimental feed to assess if the target concentration was achieved and to determine the homogeneity. In this way, samples were collected at the beginning, middle and at the end of the mixing stream, in order to monitor the whole mixing cycle. The samples were kept at room temperature ($t_{R,}$ 15-25 °C) until analysis (within a time frame of 2 weeks).

3.4 ANIMAL EXPERIMENT

Twenty-four pigs with an average BW of 27.0 ± 4.0 kg were randomly divided into 4 equal groups (3 males and 3 females/group): one control group and three experimental groups (**Figure 19a**). Each group was housed in a strictly separated (no airborne contact) 3 by 4 meter pen with a concrete floor and natural light cycle. The temperature varied between 21 °C and 25 °C. The floor was cleaned with water every day just before sample collection. *Ad libitum* access to drinking water and feed was provided throughout the experiment. After a one-week acclimatization period, each experimental group received during ten days experimental feed containing 3% cross-contamination levels of either CTC, DOX or SDZ-TRIM. The control group received blank feed (no antimicrobials). Individual faecal samples were taken by rectal stimulation, just before the start of providing the experimental diets and at day 2, 4, 6, 8 and 10 of the experimental feeding period (**Figure 19b**). In case no individual sample could be obtained (which was the case for in total 19 time points from 12 pigs), fresh faecal samples were collected from the cleaned floor. On day 11, all animals were euthanized through a combined intramuscular injection of xylazine (4.4 mg/kg BW), zolazepam and tiletamine (both 2.2 mg/kg BW) followed by

an intracardial injection of 0.3 ml/kg BW of T61® (MSD, Brussels, Belgium). Immediately after euthanasia, samples of caecal content and colonic content from different sampling segments [proximal colon ascendens (PCA), distal colon ascendens (DCA), colon descendens (CD)] were taken from each animal individually. The samples were directly stored at -80 °C without homogenization.

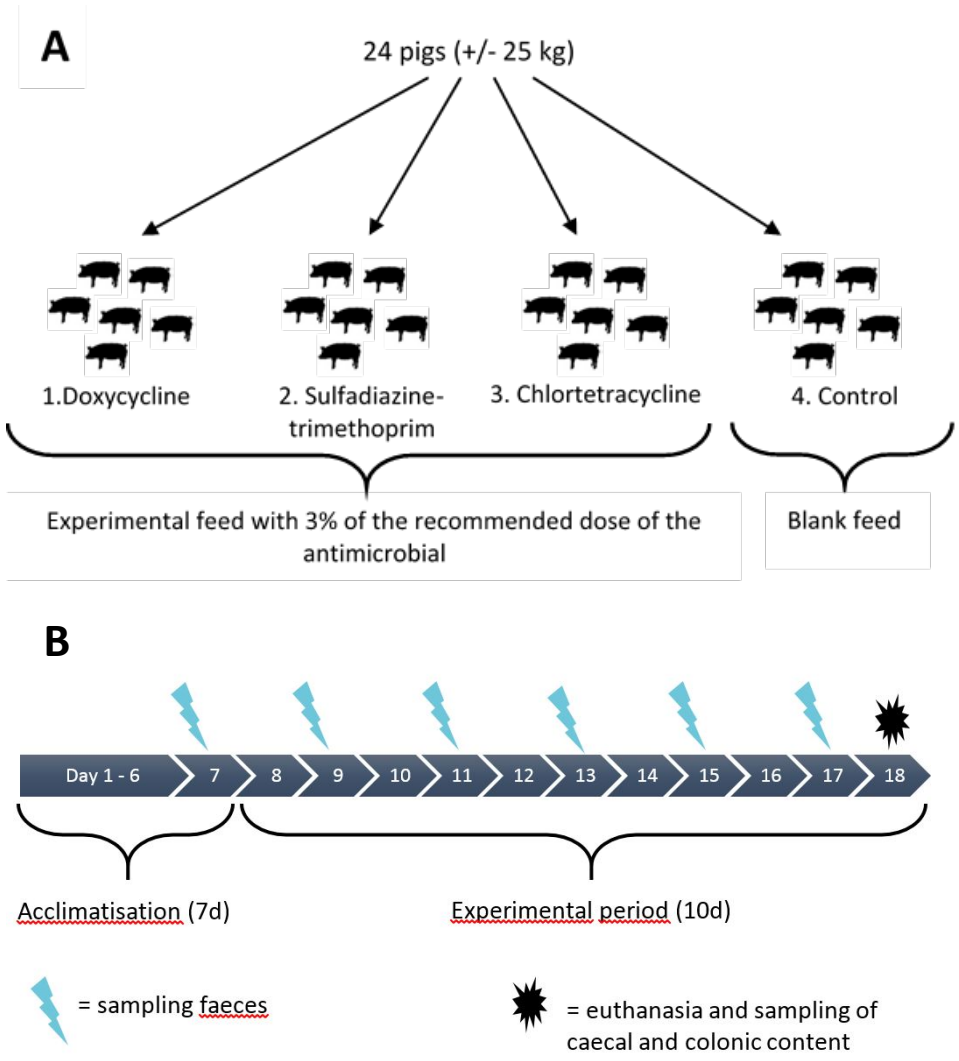


Figure 19. Experimental setup of the animal experiment. **(A)** Grouping of the animals. **(B)** Chronological scheme of the experiment.

3.5 QUANTITATION OF ANTIMICROBIALS IN FEED AND FAECES

In-house developed methods were applied for both analysis of tetracyclines (Cherlet *et al.* 2006, Cherlet *et al.* 2003) and SDZ-TRIM (Vandenberge *et al.* 2012a, Vandenberge *et al.* 2012b).

a) Tetracyclines analysis

Twenty-five ml of CH_3OH were added to 5.0 g of each feed sample. After 20 min of shaking on an in-house rotary shaker, samples were centrifuged (6261 *g*, 10 min, 4 °C). Two-hundred µl of supernatant were transferred into an Eppendorf tube and 800 µl of CH_3OH were added. After adding 50 µl of IS (20 µg/ml), samples were vortex mixed. Next, the samples were filtered through a polyvinylidene fluoride membrane (PVDF, 0.22 µm Millex-GV, Millipore, Overijse, Belgium) and transferred to an autosampler vial and 5 µl was injected onto the liquid chromatography – tandem mass spectrometry (LC-MS/MS) instrument.

To 2.0 g of intestinal content or faeces, 50 µl of IS (100 µg/ml) were added. After vortex mixing (15 s), 10.0 ml of sodium succinate solution (0.1 M) were added and the samples were again vortex mixed (15 s). Samples were then shaken (20 min, in-house rotary shaker) and centrifuged (6261 *g*, 10 min, 4 °C). The supernatant was transferred to a new plastic tube and vortex mixed (15 s) after adding 1.0 ml of 20% CCl_3COOH. These tubes were centrifuged again (6261 *g*, 10 min, 4 °C) and the samples were filtered through a Whatman filter (Whatman n°541, VWR, Leuven). This filtrate was used for further solid-phase clean-up. After preconditioning an OASIS® HLB (hydrophilic-lipophilic balance) 60 mg/3 ml solid phase extraction column (Waters, Milford, MA, U.S.) with consecutively 3 ml of CH_3OH, 3 ml of HCl (1 M) and 3 ml of HPLC (high-performance liquid chromatography) water, the filtrate was poured onto the HLB column. The

column was then washed with 1 ml of water and dried. The analytes were eluted with 3 ml of CH_3OH. The eluate was passed through a PVDF filter, transferred to an autosampler vial and 5 µl was injected onto the LC-MS/MS instrument.

The LC system consisted of an Acquity autosampler and an Acquity binary solvent manager from Waters (Milford, U.S.). Chromatographic separation was achieved on an Acquity UPLC (ultra performance liquid chromatography) BEH C18 column (50 mm x 2.1 mm i.d., 1.7 µm) from Waters. The temperatures of the autosampler tray and column oven were set at 10 °C and 30 °C, respectively. Mobile phase A consisted of CH_3CN whereas mobile phase B was 0.1% HCOOH in water. Flow rate was set at 0.3 ml/min and the following elution program was run: 0-4.0 min (10% A), 4.0-5.0 min (linear gradient to 90% A), 5.0-7.1 (90% A), 7.1-7.2 (linear gradient to 10% A), 7.2-9.0 min (10% A). The detection was performed with a Quattro Premier XE triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) probe operating in the positive ionization mode (Waters). Masslynx software v 4.1 was used to quantitate, based on the following MS-MS transitions: *m/z* 479.04 > 461.84 (CTC) and *m/z* 445.10 > 427.96 (DOX).

b) Sulphadiazine-trimethoprim analysis

After homogenization of the feed sample, 5.0 g of feed was weighed and 50 µl of each IS (1 mg/ml) and 25 ml of CH_3OH were added. The sample was vortex mixed, shaken on a horizontal shaker (Edmund Bühler, Hechingen, Germany) during 30 min, and centrifuged (4000 *g*, 15 min, t_R). Five ml of the supernatant were evaporated to dryness at 45 ± 5 °C under nitrogen. The sample was redissolved in 10 ml of CH_3CN/water (50/50 V/V), vortex mixed (30 s), diluted to 1/15 in CH_3CN/water (50/50, V/V), vortex mixed (30 s) and transferred to an autosampler vial.

For intestinal content or faeces analysis, 2.0 g of sample was weighed after homogenization and 40 µl of each IS (1 µg/ml) were added. The sample was carefully mixed with 8 g of Na_2SO_4 with a spatula to obtain a dry mixture. If necessary, extra Na_2SO_4 was added until the sample was dry. After adding 10 ml of CH_3CN, the sample was vortex mixed, shaken during 30 min (horizontal shaker, Edmund Bühler) and centrifuged (15 min, 4000 *g*, t_R). Five ml of the supernatant were then transferred into a glass tube and evaporated to dryness under nitrogen in a water bath of 45 °C. Next, the sample was redissolved in 1 ml of an CH_3CN/water mixture (50/50, V/V) containing 0.1% CH_3COOH in water, vortex mixed (30 s), sonicated (5 min), and filtered through a PVDF filter into an autosampler vial.

Chromatographic separation was performed on a Waters Acquity UPLC system. An Acquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm) was used and the analysis was performed with a gradient of water/CH_3CN (95/5, V/V) + 0.3% CH_3COOH (mobile phase A) and water/CH_3CN (5/95, V/V) + 0.3% CH_3COOH (mobile phase B). The following elution program was run: 0-8 min (100% A), 8-12 min (70% A), 12-13 min (0% A), 13-13.01 min (linear gradient to 100% A), 13.01-14.6 min (100% A). Flow rate was set at 0.4 ml/min. A Xevo TQ-MS triple quadrupole mass spectrometer with an ESI (electrospray ionisation) probe operating in the positive ionization mode was used. Quantitation was done with Masslynx software v 4.1. MS-MS transitions for SDZ were: *m/z* 250.89 > 155.94/107.96 and for TRIM: *m/z* 290.98 > 122.99/230.01. The detected ion ratio's for the different samples were within the permitted tolerances specified in Commission Decision 2002/657/EC (EC 2002).

3.6 METHOD VALIDATION

The methods were validated for feed and faeces according to a set of parameters that were in compliance with the recommendations and guidelines defined by the European Community (EC 2002) and international standards for validation of analytical methods in residue depletion studies. The following set of parameters was determined: limit of detection (LOD, n=6), limit of quantification (LOQ, n=6), linearity (R^2 and goodness-of-fit coefficient (g)), precision (repeatability, RSD_r (n=6), and reproducibility, RSD_R (n=6), RSD = relative standard deviation) and trueness (n=6). Validation samples were prepared with blank feed from the same batch as the feed that was administered during the experiment and blank faeces were obtained from pigs that were not treated with antimicrobial drugs.

3.7 STATISTICAL ANALYSIS

After determination of normality and homogeneity of variances, one-way analysis of variance (ANOVA) (SPSS 22, IBM, Chicago, IL, U.S.) was performed for each antimicrobial on the concentrations from the four different intestinal segments. A Scheffé test was performed as post-hoc test. The significance level was set at 0.05.

4 RESULTS

4.1 METHOD VALIDATION

The results of the method validation are given in supplementary **Table S1**. All values, except for the trueness in case of SDZ in feed, were within the acceptance ranges according to Commission Decision 2002/657/EC.

4.2 CONCENTRATIONS IN EXPERIMENTAL FEED

Ten samples of each batch of experimental feed were analyzed to assess if the target concentrations (3% of the MRD) were achieved. Mean concentrations ± standard deviation (SD) in the feed were 7.33 ± 6.87 mg/kg (= 3.26% of MRD) for DOX, 9.98 ± 5.35 mg/kg (= 3.23% of MRD) for CTC, 12.99 ± 4.15 mg/kg (= 3.12% of MRD) for SDZ and 2.31 ± 0.90 mg/kg (= 2.77% of MRD) for TRIM. In all experimental diets, there was a high variation between sample concentrations.

4.3 CONCENTRATIONS IN FAECES

The mean (+ SD) concentrations of CTC, DOX and SDZ in the faeces are shown in **Figure 20**. A steady-state was reached around day 4 for CTC (± 10 mg/kg), DOX (± 4 mg/kg) and SDZ (± 0.7 mg/kg). Concentrations of TRIM were very low; all results except two were lower than the LOD of 0.016 mg/kg. No traces of antimicrobials were found in the faecal samples taken on day 0, just before the start of the experimental period.

Transfer ratio's (TR), i.e. the mean concentration in faeces taken over day 4 – day 10 of the experimental period, and divided by the mean concentration in feed, were 102.5%, 55.4% and 4.7% for CTC, DOX and SDZ, respectively (**Table 7**, p. 108).

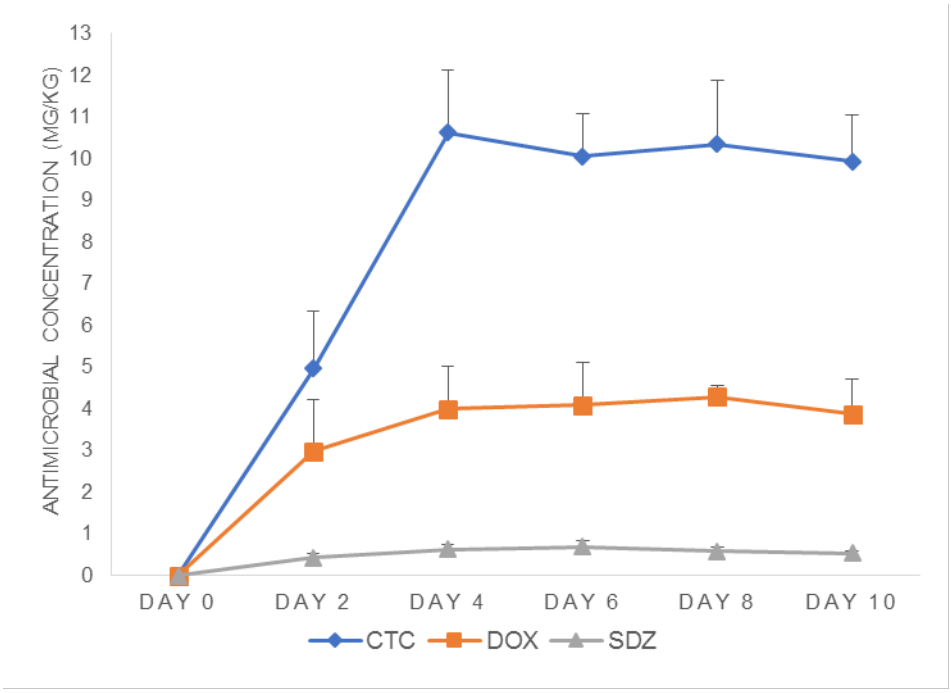


Figure 20. Mean concentrations (mean of six pigs + standard deviation) of chlortetracycline (CTC), doxycycline (DOX) and sulphadiazine (SDZ) in pig faeces during 10 days of ad libitum feeding with feed containing 3% cross-contamination levels of these antimicrobials. For CTC, concentrations rose from 4.97 mg/kg to a steady-state of approximately 10 mg/kg. Mean concentrations of DOX rose from 2.99 mg/kg to a steady-state of approximately 4 mg/kg. Mean concentrations of SDZ rose from 0.44 mg/kg to a steady-state of approximately 0.70 mg/kg.

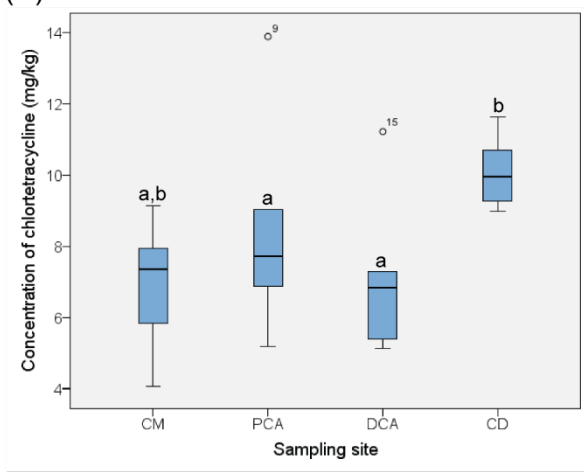
4.4 CONCENTRATIONS IN CAECAL AND COLONIC CONTENT

CTC, DOX and SDZ concentrations in caecal content and contents of different segments of the colon after 10 days of feeding are presented in **Figure 21**. Min/max/mean concentrations for CTC in the different intestinal segments were 4.06/9.15/6.95 mg/kg (caecum, CM), 5.20/13.89/8.41 mg/kg (PCA), 5.14/11.22/7.12 mg/kg (DCA) and 8.99/11.63/10.08 mg/kg (CD). For DOX, these concentrations were 1.01/3.07/1.78 mg/kg (CM), 1.47/5.86/3.31

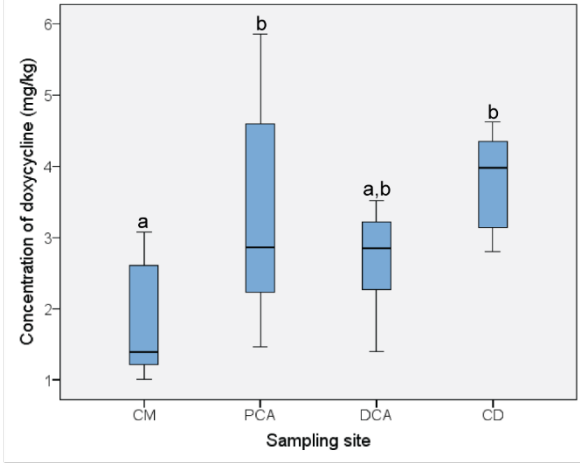
mg/kg (PCA), 1.40/3.51/2.68 mg/kg (DCA) and 2.80/4.62/3.81 mg/kg (CD). SDZ concentrations were 0.23/0.83/0.47 mg/kg (CM), 0.21/0.67/0.45 mg/kg (PCA), 0.51/1.00/0.67 mg/kg (DCA) and 0.47/0.65/0.54 mg/kg (CD). All results for TRIM were again lower than the LOD of 0.016 mg/kg. Concentrations in the CD approached the average faeces concentration found for CTC, DOX and SDZ. CTC concentrations found in the CD proved to be significantly higher compared to concentrations in the DCA and the CM, but not compared to the PCA. DOX levels in the CM were significantly lower than in the PCA and CD. SDZ levels in the DCA were significantly higher than in the PCA. The TR's, i.e. the mean concentration in CM, PCA, DCA or CD divided by the mean concentration in the feed, are given in (**Table** 7, p. 108).

Figure 21 (right). Concentrations of A) chlortetracycline (CTC), B) doxycycline (DOX) and C) sulphadiazine (SDZ) in caecal content and colonic content from three different sampling segments (6 indepentent observations per sampling segment). Samples were taken from 6 pigs per group after 10 days of *ad libitum* feeding with feed containing 3% cross-contamination levels of CTC, DOX and SDZ. Mean concentrations in caecum (CM), proximal colon ascendens (PCA), distal colon ascendens (DCA) and colon descendens (CD) were 6.95, 8.41, 7.12 and 10.08 mg/kg (CTC), 1.78, 3.31, 2.68 and 3.81 mg/kg (DOX) and 0.47, 0.45, 0.67 and 0.54 mg/kg (SDZ), respectively. The two outlying values for CTC (observation 9 and 15) belong to one animal. A different letter (a or b) denotes a significant difference in concentration between sampling segments ($p < 0.05$).

(A)


Concentration of chlortetracycline (mg/kg)
14
12
10
8
6
4
9
15
a,b
a
a
b
CM
PCA
DCA
CD
Sampling site

(B)


Concentration of doxycycline (mg/kg)
6
5
4
3
2
1
a
b
a,b
b
CM
PCA
DCA
CD
Sampling site

(C)

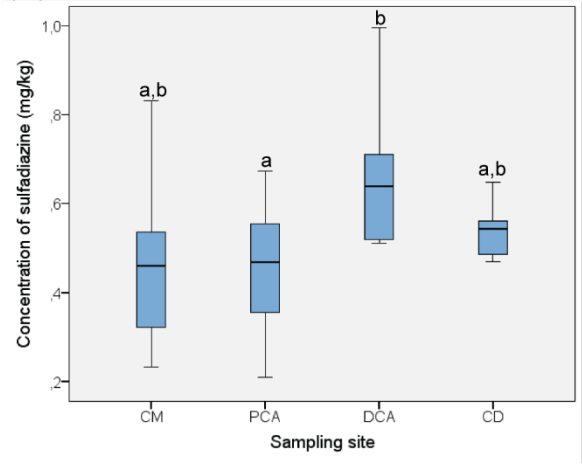

Concentration of sulfadiazine (mg/kg)
1,0
,8
,6
,4
,2
a,b
a
b
a,b
CM
PCA
DCA
CD
Sampling site

Table 7. Oral bioavailability (F) compared to transfer ratio's (TR) of chlortetracycline (CTC), doxycycline (DOX) and sulphadiazine (SDZ)

Antimicrobial	F	TR				
		CM	PCA	DCA	CD	faeces
CTC	6% [a]	69.6%	84.2%	71.4%	101.0%	102.5%
DOX	21-50% [b]	24.3%	45.1%	36.6%	51.9%	55.4%
SDZ	85-100% [c]	3.7%	3.5%	5.2%	4.2%	4.7%

TR's are calculated by dividing the mean concentration (6 pigs) in the content of an intestinal segment (caecum, CM; proximal colon ascendens, PCA; distal colon ascendens, DCA; colon descendens, CD) after 10 days of feeding by the mean concentration in the feed. The mean concentration in the faeces was taken over day 4 - day 10 (steady-state).

[a] Nielsen *et al,*. 1996.

[b] Baert *et al*., 2000, Sanders *et al.* 1996.

[c] Baert *et al.*, 2001; Nielsen *et al.*, 1994.

5 DISCUSSION

The aim of this study was to determine which concentrations of CTC, DOX, SDZ and TRIM could be found in intestinal content and faeces of pigs when feed containing a 3% carry-over concentration was administered.

In each experimental diet the target concentration of 3% of the MRD was approached (2.77 – 3.26%). Although the best possible sampling procedure (Borras *et al.* 2011) was applied, a large variation between the samples was found. Adequate mixing of the premixes depends on multiple factors, including physico-chemical properties such as particle size and electrostatic properties of the premix. Other factors that influence homogeneity are the composition of the final feed, type of mixing machinery, mixing in stages or trituration and precision and size of the samples taken for analysis (EMEA 1996). Since very small volumes of premix had to be mixed with large amounts of blank feed, it is not surprising that a large variability among samples was found. Moreover, studies on cross-contamination in feed mills show that antimicrobials are not homogenously divided in flushing feed either (Stolker *et al.* 2013). In contrast, concentrations found in intestinal content and faeces showed a much lower variation.

In case of SDZ-TRIM, concentrations found in faeces and intestinal content were very low (SDZ max. 0.995 mg/kg, TRIM < LOD). Except for two values, all results for TRIM were lower than the LOD. Since the administered dose was very low (2.31 mg/kg feed), the reported oral bioavailability for TRIM is high [73-92% (Baert *et al.* 2001, Nielsen *et al.* 1994)] and elimination occurs through renal excretion, very low intestinal concentrations were indeed expected. On the other hand, quantitative results for SDZ were obtained, although the oral bioavailability of SDZ in pigs is even

higher than for TRIM. This can be explained by the higher absolute dosage of SDZ (12.99 mg/kg feed) compared to TRIM, as both compounds are present in a 5/1 ratio (SDZ/TRIM) in the used formulation. Interestingly, the calculated TR's from feed to gut of SDZ (3.5-5.2%) correspond well to the high oral bioavailability reported in pigs [85-100% (Baert *et al.* 2001, Nielsen *et al.* 1994)], i.e. the higher the oral bioavailability the lower residual concentrations in the gut can be expected unless extensive biliary excretion or secretion in the gut takes place.

The concentrations of tetracyclines in faeces and caecal and colonic content were found to be relatively high. In general, higher concentrations were seen in the distal part of the colon compared to the proximal part and the caecum. The main explanation for these observations is probably the progressive absorption of water throughout the intestinal tract. As in the case of SDZ, the calculated TR's from feed to gut (CTC 69.6-102.5%, DOX 24.3-55.4%) correspond well to the reported oral bioavailability in pigs [CTC 6%, DOX 21-50% (Baert *et al.* 2000, Nielsen *et al.* 1996, Sanders *et al.* 1996)]. It has to be taken into account though, that the bioavailability of tetracyclines is highly variable, most likely due to presence of feed in the gastrointestinal tract. It is known that oral absorption of tetracyclines may be reduced in the presence of bivalent ions (Baert *et al.* 2000, Santos *et al.* 1996). Also, the study design to calculate oral bioavailability's may vary between different studies, e.g. the prandial state of the animals. Taking into account that our experiment involves feed administration, references regarding oral bioavailability in non-fasted pigs in particular were consulted. Especially for DOX, the oral bioavailability seems to vary, even within the same study between individual animals [8.0-32.4% (Baert *et al.* 2000), 40-50% (Sanders *et al.* 1996)]. The intestinal concentrations of DOX in the present study correspond best to the oral bioavailability reported in a previous study that also used a premix formulation [40-50% (Sanders *et al.*

1996)] when compared to administration of an oral powder [8-32.4% (Baert *et al.* 2001)]. Besides oral bioavailability, also the excretion route can influence the intestinal concentrations of a drug. SDZ-TRIM and CTC are renally excreted whereas up to 75% of DOX is excreted unchanged in the intestinal tract (Ahrens *et al.* 2008). It would therefore be expected that the transfer ratio (TR) of DOX is higher than based solely on oral bioavailability. The large variability in reported oral bioavailabilities for DOX might explain the relatively low TR indicating that this TR only serves as a guidance value and depends on several factors.

The oral bioavailability of a drug is usually determined for its therapeutic dose. Given the inverse relation found between the oral bioavailability and intestinal concentrations of SDZ, CTC and DOX, it is rather likely that there is a linear relation between the administered dose and intestinal concentrations. This information can be used in the risk assessment of different cross-contamination levels of pig feed regarding potential resistance selection in the intestinal microbiota. However, this conclusion can only be drawn for the tested antimicrobials and animal species. Furthermore, additional experiments should be performed to confirm our results - ideally testing a range of antimicrobial concentrations - as there is no previous research available to compare. A non-peer reviewed report though, estimated intestinal concentrations of CTC to be 1.68 mg/kg in case of administration of 12 mg CTC/kg feed. This is clearly lower compared to our results (min 5.1- max 13.9 mg/kg CTC in colonic content with 9.98 mg CTC/kg feed) (Burch 2011).

In a recent study, manure samples obtained from different pig, poultry and veal calve farms in the Netherlands were examined for the presence of antimicrobial residues (Zuidema *et al.* 2014). In 16 out of 20 of the sampled pig farms, residues were detected although no recent use of antimicrobials was reported. Tetracyclines were found in 14 of these farms, with DOX

concentrations ranging from 2 µg/kg to 95,000 µg/kg. Sulphonamides were detected in 6 out of 20 farms, with SDZ concentrations ranging from 1 µg/kg to 216 µg/kg. In light of these data, it is clear that one should not focus on the absolute results based on 3% carry-over levels obtained in this study, but rather on the relation found between the oral bioavailability of CTC, DOX and SDZ and intestinal concentrations.

In recent years, more attention has been paid to the possible effects of low antimicrobial concentrations on selection of resistant bacteria. Pioneer studies revealed important effects of very low concentrations on resistance selection *in vitro*. Gullberg and co-authors performed competition experiments between strains resistant and susceptible to tetracycline and found minimal selective concentrations of 15 ng/ml (competition between isogenic - except for the resistance determinant - *Salmonella* Typhimurium strains, Gullberg *et al.* 2011 and 45 ng/ml tetracycline (competition between isogenic *E. coli* strains, with or without resistance plasmid pUUH239.2, Gullberg *et al.* 2014). The minimal selective concentration was in this case defined as the concentration where the fitness cost of the resistance is balanced by the antimicrobial-conferred selection for the resistant mutant. This would mean that even concentrations of tetracyclines 100x lower than those found in this study can cause resistance selection. Brewer and co-authors (2013) investigated the effect of 1 µg/ml of different antimicrobials on transfer of resistance genes *in vivo* in pigs and found that 1 µg/ml of tetracycline and sulphamethazine increased transfer frequency, whereas 1 µg/ml of sulphathiazole did not. It is likely that intestinal concentrations of 1 µg/ml of tetracyclines can be found in pigs, considering our results and the maximum allowed carry-over level (1%) established in the Belgian covenant (BFA and FASFC 2013).

6 CONCLUSIONS

This study showed an inverse relation between intestinal concentrations and the oral bioavailability for SDZ-TRIM as well as for CTC and DOX, which have a high, respectively low oral bioavailability in pigs. As different studies (Stolker *et al.* 2013, Zuidema *et al.* 2014) indicate there is a large variation in cross-contamination levels of feed, this result can be an important tool to evaluate possible risks of different contamination levels. Further research is needed to determine the effect on resistance selection in the intestinal microbiota. Furthermore, it would be interesting to perform additional experiments, confirming our results and analysing other antimicrobials that are used as premix formulation, such as penicillins, macrolides, pleuromutilins and polymyxins (BelvetSAC 2015).

7 ACKNOWLEDGEMENTS

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8 SUPPLEMENTARY INFORMATION

Table S1 (next page).

Table S1. Validation parameters for quantification of chlortetracycline (CTC), doxycycline (DOX), sulphadiazine (SDZ) and trimethoprim (TRIM) in pig feed and faeces

Matrix	Analyte	Validation levels (feed: mg/kg; faeces: µg/kg)	LOD (feed: mg/kg; faeces: µg/kg) N=6	LOQ (feed: mg/kg; faeces: µg/kg) N=6	Linearity (R^2)	Goodness-of-fit coefficient (g)	RSD_r (%) N=6	RSD_R (%) N=6	Trueness (%) N=6
Feed	CTC	4.65 / 9.29 / 11.63	0.47	4.65	0.9959	3.96	12.2 / 6.9	n.d. / n.d. / 4.0	96.1 / 85.9 / 99.1
	DOX	3.61 / 6.76	0.09	3.61	0.9974	2.95	9.3 / 8.7	n.d.	95.4 / 89.4
	SDZ	7.0 / 15.0	1	2	0.9946	9.0	8.1 / 4.0	n.d.	139.9* / 117.9*
	TRIM	7.0 / 15.0	0.25	0.5	0.9968	19.0	2.6 / 1.9	n.d.	97.0 / 105.4
Faeces	CTC	1,000 / 4,500	34.4	1,000	0.9989	2.96	6.6 / 3.9	n.d. / 6.3	98.2 / 105.4
	DOX	1,000 / 4,500	11.5	1,000	0.9959	6.11	6.5 / 2.9	n.d. / 10.6	106.4 / 103.2
	SDZ	100 / 250 / 500	33.0	67.0	0.9929	19.0	9.1 / 11.5 / 5.1	8.6 / 11.4 / 7.6	94.6 / 97.9 / 98.0
	TRIM	100 / 250 / 500	16.0	32.0	0.9947	13.0	7.2 / 6.3 / 2.7	6.1 / 8.1 / 6.4	100.7 / 101.1 / 102.5

Acceptance criteria (EC 2002): trueness 80-110%, $RSD_{r\ max}$: 4.65 mg/kg: 14.1%, 9.29 mg/kg: 12.7%, 11.63 mg/kg: 18.4% (CTC); 3.61 mg/kg: 12.7%, 6.76 mg/kg: 11.5% (DOX),7.0 mg/kg: 7.9%, 15.0 mg/kg: 7.1% (SDZ, TRIM), 1,000 µg/kg: 10.7%, 4,500 µg/kg: 8.5% (CTC, DOX), 100 µg/kg: 15.1%, 250 µg/kg: 13.1%, 500 µg/kg: 11.9% (SDZ, TRIM). LOD, limit of detection; LOQ, limit of quantification; RSD_r, repeatability, RSD_R, within laboratory reproducibility; n.d., not done; *: criterium not fulfilled

CHAPTER 4

Effect of residual doxycycline concentrations on resistance selection and transfer in porcine commensal *escherichia coli*

Adapted from:

Peeters, L. E. J., Croubels, S., Rasschaert, G., Imberechts, H., Daeseleire, E., Dewulf, J., Heyndrickx, M., Butaye, P., Haesebrouck, F. and Smet, A. (2018). Effect of residual doxycycline concentrations on resistance selection and transfer in porcine commensal *Escherichia coli*. *International Journal of Antimicrobial Agents*, 51(1):123-127.

1 ABSTRACT

Pig feed may contain various levels of antimicrobial residues due to cross-contamination. A previous study showed that a 3% carry-over level of DOX in the feed results in porcine faecal concentrations of approximately 4 mg/l. Resistant bacteria, enriched in the pig's intestinal microbiota due to these residual DOX concentrations, could cycle between different environmental, animal and human reservoirs and transfer their genes to pathogenic bacteria.

This study aimed to determine the effect of residual DOX concentrations (1 and 4 mg/l) *in vitro* on selection of DOX resistant porcine commensal *E. coli* and transfer of their resistance plasmids.

Three DOX resistant porcine commensal *E. coli* strains and their plasmids were characterized. These strains were each brought in competition with a susceptible strain in a medium containing 0, 1 and 4 mg/l of DOX. Resistant bacteria, susceptible bacteria and transconjugants were enumerated after 24h and 48h.

The *tet*(A) carrying plasmids showed genetic backbones that are also present among human *E. coli* isolates. Ratios of resistant to susceptible bacteria were significantly higher at 1 and 4 mg/l DOX compared to the blank control, but no significant difference between 1 and 4 mg/l was seen. Plasmid transfer frequencies were affected by 1 or 4 mg/l DOX in the medium for only one of the resistance plasmids.

In conclusion, DOX concentrations of 1 and 4 mg/l can select for resistant *E. coli in vitro*. Further research is needed to determine the effect of these concentrations in the complex environment of the porcine intestinal microbiota.

2 INTRODUCTION

As antimicrobial resistance represents a global threat to human and animal health, the understanding of its mechanisms and drivers is of major importance (Holmes *et al.* 2016). Conventionally it was assumed that selection of resistant bacteria is promoted at concentrations between the MIC of the susceptible population and the MIC of the resistant population of the bacterial species (Drlica 2003). The past decade however, the possible impact of the widely reported presence of antimicrobial residues in different environments gained attention (Andersson *et al.* 2014). Antimicrobial residues may enter the environment through various routes and cross-contamination of pig feed through spoilage has been shown to be one of them (Stolker *et al.* 2013). Administration of antimicrobial drugs through feed medication is frequently applied in the pig industry (BelVetSAC 2016, EMA 2016). These medicated feeds may cause cross-contamination of non-medicated feed at the feed mill, during transport and/or at farm level (Filippitzi *et al.* 2016, Stolker *et al.* 2013). Tetracyclines accounted for the most frequently sold veterinary antimicrobials (33.4%) in Europe in 2014 (EMA 2016). Furthermore, approximately 68% of the tetracyclines sold in Belgium in 2015 was represented by DOX (BelVetSAC 2016). In a previous *in vivo* study (Chapter 3), the intestinal and faecal concentrations of DOX, chlortetracycline and sulphadiazine-trimethoprim, were determined in pigs. The animals were administered feed containing 3% carry-over levels of these antimicrobials, based on the maximum allowed carry-over level according to the European Commission Directive of 2009 (EP 2002). In 2013, however, a covenant was established in Belgium stating that cross-contamination with antimicrobials should not exceed 1% of the recommended dose (BFA and FASFC 2013). Therefore, the results obtained for feed with 3% cross-contamination were in the present study also extrapo-

lated to a 1% cross-contamination level. The *in vivo* study showed that especially tetracyclines, with a low to moderate oral bioavailability in pigs, can be found in residual concentrations in caecum and colon (Chapter 3). These unintended concentrations possibly exert a selective pressure on tetracycline resistant bacteria present in the intestinal microbiota and may co-select for other resistance genes carried by these bacteria (Gullberg *et al.* 2014). Subsequently, pork can become contaminated with bacteria that might carry resistance genes against antimicrobials which are used in human medicine (e.g. β-lactams). Consumption of raw or undercooked meat may allow the spread of these bacteria to the human gut, where they could transfer their resistance genes to human commensals and pathogens, e.g. *Salmonella enterica* and *Escherichia coli* O157:H7 (Van Meervenne *et al.* 2012). Also contamination of the environment through faeces or dust might result in indirect contact between animals and humans. Hence, the frequently applied tetracyclines in pig feed such as DOX may indirectly impose a risk for human health. Indeed, very low concentrations of tetracycline and/or oxytetracycline ranging from 1 to 1000 µg/L have already been shown to select for resistant bacteria or genes *in vitro* (Gullberg *et al.* 2014, Gullberg *et al.* 2011, Johnson *et al.* 2015) and in different environments such as biofilms (Lundstrom *et al.* 2016), arable soils (Shentu *et al.* 2015), and the intestinal microbiota of dairy calves (Pereira *et al.* 2014).

The present study aimed to investigate the selective pressure on DOX resistant *E. coli* of 4 mg/l and 1 mg/l of DOX, corresponding with intestinal DOX concentrations caused by a cross-contamination level of the feed of approximately 3% and 1%, respectively. Whereas previous research on selective pressure at residual concentrations involved among others competition experiments between isogenic lab strains (Gullberg *et al.* 2014, Gullberg *et al.* 2011), mathematical models (Bengtsson-Palme *et al.* 2016), or *in vivo* studies (Johnson *et al.* 2015, Pereira *et al.* 2014), *E. coli* field strains

isolated from pig faeces were preferred as model bacteria for the pig microbiota in this study.

3 MATERIAL AND METHODS

3.1 BACTERIAL STRAINS

The *E. coli* strains used in this study originated from a collection that was obtained between 2011 to 2013 for an antimicrobial resistance monitoring program of the Belgian Federal Agency for the Safety of the Food Chain (Hanon *et al.* 2015). Three tetracycline resistant commensal *E. coli* strains (EC 682, EC 202, EC 292) isolated from pig faeces were selected to be used as donor strains in competition experiments. MIC values of these *E. coli* isolates were determined for ampicillin, chloramphenicol, ciprofloxacin, colistin, florphenicol, cefotaxime, gentamicin, kanamycin, nalidixic acid, sulphamethoxazole, streptomycin, tetracycline and trimethoprim using Sensititre micro broth dilution (TREK Diagnostic Systems, West Sussex, UK) according to EU Reference Laboratory for antimicrobial resistance (EURL-AR 2013) guidelines. One strain (EC 682) showed the most prevalent phenotypic resistance profile (**Table 8**). The MIC of DOX for the three donor strains was determined by the broth microdilution method according to the CLSI document M07-A10. Presence of common tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(G) was verified by PCR assays (Ng *et al.* 2001).

From the same collection, three *E. coli* strains (EC 298, EC 400, EC 588) susceptible to all antimicrobials tested, were selected as recipient strains and to compete with the donor strains.

As bacterial fitness plays a major role in competition between strains, bacterial growth curves were set up for each strain separately, in three different conditions (0, 1 and 4 mg/l DOX), using an automated microbiology growth curve analysis system (Infinite 200Pro, Tecan Group Ltd., Männedorf, Switzerland). Briefly, in each well of a 96-well plate, 100 µL of an over-

night bacterial culture in Miller's LB broth (LB) of approximately 10^5 colony forming units (CFU)/ml was added to 100 µL of LB with a final concentration of 0, 1 or 4 mg/l DOX, respectively. The plate was incubated at 37°C during 18h and every 5 min OD_{600} (optical density at wavelength 600nm) was measured. Each curve was done in triplicate. The growth rate was calculated manually as described by Hall *et al* (Hall *et al.* 2013).

3.2 CONJUGATION EXPERIMENTS AND PLASMID ANALYSIS

The mobility of the DOX resistance conferring plasmids (DOX^R plasmids) in the selected donor strains was first verified under blank standard conditions with as acceptor a rifampicin (RIF) resistant *E. coli* lab strain (J5RIF) (Bertrand *et al.* 2006). Conjugation experiments were performed overnight in LB at 37°C with a donor/recipient ratio of 1:5. Transconjugants were grown selectively on MacConkey n°3 agar (MC, Oxoid Ltd, Basingstoke, UK) with filter sterilized solutions of 8 mg/l DOX and 80 mg/l RIF (both from Sigma-Aldrich, Bornem, Belgium).

A disk diffusion test was performed in triplicate on each transconjugant to verify if co-transfer of resistance determinants occurred. Genomic DNA from the *E. coli* J5 transconjugants obtained by a standard boiling method was used to define the Incompatibility group of the DOX^R plasmids (Carattoli *et al.* 2005).

The three susceptible strains were checked for the presence of replicons of the same type as the donor plasmids using the same method as described above. Subsequently, standard conjugation experiments for each donor strain were conducted to confirm if the three mobile plasmids could be transferred to the susceptible strains.

Finally, DNA from the DOX^R plasmids, extracted with a Qiagen (Antwerp, Belgium) plasmid midi kit (manufacturer's protocol), was checked for purity by gel electrophoresis and sent to Baseclear B.V. for sequencing

purposes (Leiden, The Netherlands). Briefly, Illumina genomic Nextera XT library preparation was followed by Illumina HiSeq 2500 sequencing. FASTQ sequences were trimmed and assembled using CLC Genomics Workbench version 8.0. The scaffolds were annotated using Prokka (Seemann 2014). Similar nucleotide sequences were identified using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were visualized using the BLAST Ring Image Generator (BRIG) (Alikhan *et al.* 2011).

The plasmid nucleotide sequences were deposited in the EMBL-EBI database under accession numbers FMWN01000000 (pEC202), FNLP01000000 (pEC292) and FNLQ01000000 (pEC682).

3.3 COMPETITION EXPERIMENTS

Two types of competition experiments were carried out. Experiment type 1 was performed with recipient strains EC 298 (non-conjugating strain) and EC 400 (conjugating strain). Experiment type 2 was carried out with conjugating recipient strains EC 400 and EC 588. One experiment each time involved one recipient strain that was brought in competition with each of the donor strains separately. Each experiment was repeated four times. The experimental setup is shown in **Figure 22**.

To be able to distinguish donor strains from recipient strains in mixed cultures, non-lactose fermenting mutants of the donor strains were selected (Smet *et al.* 2011). These mutants form white colonies on MC, while natural, lactose-fermenting *E. coli* form red colonies **Figure 23**.

Experiment type 1 was conducted as follows. The three donor strains and one of the recipient strains were separately grown overnight in LB broth at 37°C on a horizontal shaker (IKA KS 260 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany) and then mixed in a 1/1000 ratio (donor/recipient) in three different tubes with LB with a final volume of 30 ml and

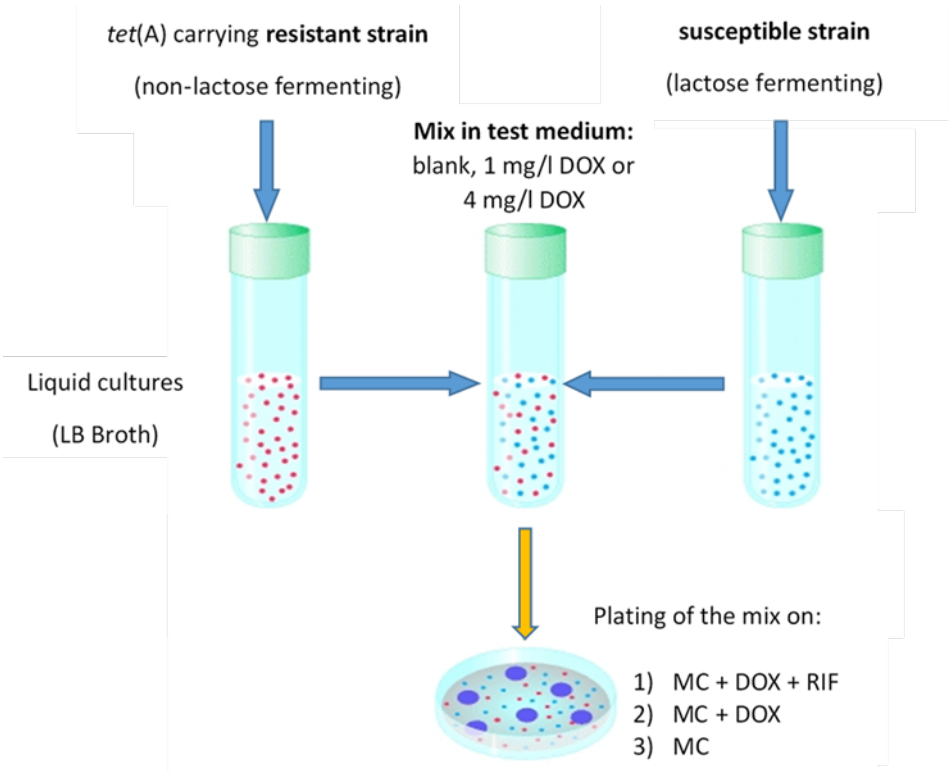


Figure 22. Setup of the competition experiments. DOX, doxycycline, LB, Luria Bertani; MC, MacConkey n°3 agar; RIF, rifampicin

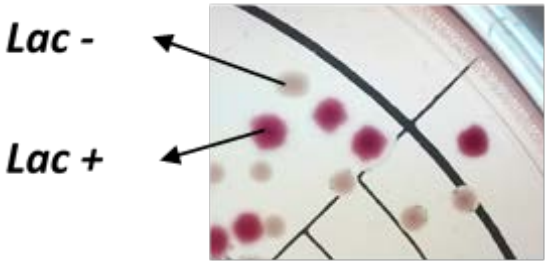


Figure 23. Morphology of lactose fermenting (Lac +) and non-lactose fermenting (Lac -) E. coli on MacConkey n°3 agar.

a final concentration of 0, 1 and 4 mg/l DOX, respectively. These cultures were incubated at 37°C during 48h on a horizontal shaker. After 24h and 48h of incubation, 1 ml was taken from each tube and appropriate dilutions were plated in duplicate or triplicate on MC with and without 8 mg/l DOX and incubated overnight at 37°C. The donor strain was enumerated by counting white colonies on MC+DOX plates and the total number of recipient bacteria was counted on MC (red colonies).

Experiment type 2 was designed to be able to enumerate transconjugants, and thus to calculate the transfer frequency of the plasmids and to count the total of resistant bacteria (donor + transconjugants) and susceptible bacteria (total recipients – transconjugants). Enumeration of transconjugants was made possible by using RIF resistant mutants (EC 400RIF and EC 588RIF) of the conjugating recipient strains. The mutants were selected by consecutive plating on MC plates with RIF gradient (0-100 mg/l) and MC plates containing 100 mg/l RIF, 150 mg/l RIF and finally 200 mg/l RIF. Preparation of the competition cultures was done as in experiment type 1. Appropriate tenfold dilutions were now plated on MC with DOX (8 mg/l) to count the donor strain, with RIF (80 mg/l) to count the recipient (total count) and with DOX (8 mg/l) + RIF (80 mg/l) to count transconjugants (DOX resistant recipient). The transfer frequency of the plasmids was calculated by dividing the number of transconjugants (CFU/ml) by the total number of recipients (CFU/ml).

The stability of DOX in LB at both 1 and 4 mg/l during 48h at 37°C was verified using LC-MS/MS. Samples of LB (30 ml) with or without DOX and with or without bacterial culture (EC 682) were taken after 0, 2, 4, 6, 7, 23, 25, 27, 29, 31, 47 and 48 hours of incubation and stored at -20°C. For the sample preparation, a 50 µL aliquot of the medium sample was spiked with 50 µL of the internal standard demethylchlortetracycline (10 µg/ml, Sigma Aldrich, Bornem, Belgium) and vortexed. Afterwards, the samples were 20

times diluted in 0.5% formic acid in water and transferred to an autosampler vial. The LC-MS/MS analysis was performed with a method described by Vandenberge and co-authors (2012a).

3.4 STATISTICAL ANALYSIS

After determination of homogeneity of the results, a linear mixed model was used to assess the effect of medium, resistant donor strain, susceptible recipient strain and time on the resistant/susceptible ratio (R/S) and transfer frequency (SPSS 23, IBM, Chicago, IL, U.S.).

4 RESULTS

4.1 CHARACTERIZATION OF BACTERIAL STRAINS

The characteristics of the lactose negative mutants of DOX resistant porcine *E. coli* strains EC 682, EC 202 and EC 292 are shown in **Table 8**. The same characterization (+ growth curve analysis) was done for the original lactose fermenting strains (data not shown) but no differences with the lactose negative mutants were observed. The MIC's of DOX for susceptible strains EC 298, EC 400, EC 400RIF, EC 588 and EC 588RIF were 1, 2, 0.5, 2 and 1 mg/l respectively. The growth curves and growth rates of all strains are given in **Figure 24**. In the absence of DOX, EC 400RIF showed a lower growth rate than EC 400. In contrast, EC 588 and EC 588RIF showed more similar growth rates. Therefore, only EC 588RIF was included in the competition experiments. Growth rates of resistant strains EC 202 and EC 292 were hardly affected by 1 or 4 mg/l DOX, while 4 mg/l DOX reduced the growth rate of EC 682 by 0.0037 min^{-1} (**Figure 24**). All susceptible strains showed a lower growth rate at 1 and 4 mg/l DOX than at 0 mg/l DOX.

Table 8. Characteristics of the doxycycline resistant *E. coli* strains (lactose negative mutants) that were used as donor strains in the competition experiments

Strain n°	Phenotypic resistance profile* (Prevalence)	Co-transferred resistance	MIC DOX	Tetracycline resistance gene	Inc-group of *tet*(A) carrying plasmid (>100kb)
EC 682	AMP-SMX-STR-TET-TMP (6.5%)	AMP-SMX-STR-TET-TMP	16 mg/l	*tet* (A)	IncI1
EC 202	AMP-CHL-STR-TET (0.3%)	CHL-STR-TET	32 mg/l	*tet* (A)	IncFII
EC 292	CHL-SMX-STR-TET-TMP (1%)	CHL-SMX-STR-TET-TMP	32 mg/l	*tet* (A)	IncFII-FIB

AMP, ampicillin; SMX, sulphonamides; STR, streptomycin; TET, tetracycline (and doxycycline); TMP, trimethoprim; CHL, chloramphenicol. The prevalences of the phenotypic resistance profiles in the 2011-2013 collection of porcine *E. coli* strains (total of 569 strains) are indicated between brackets. *According to EUCAST guidelines.

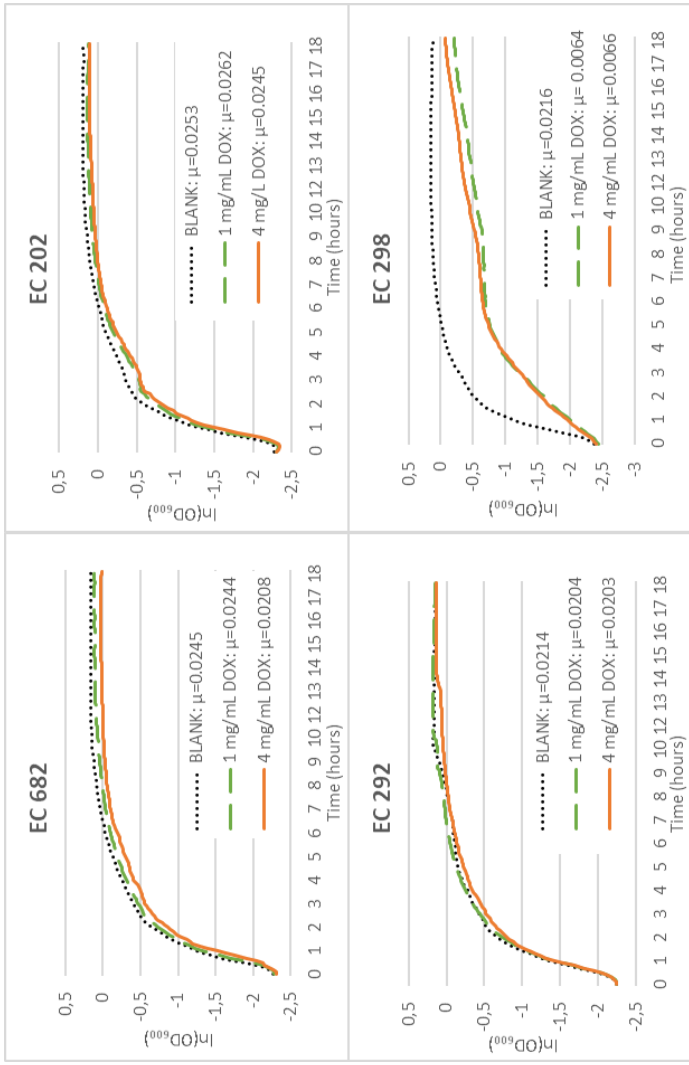
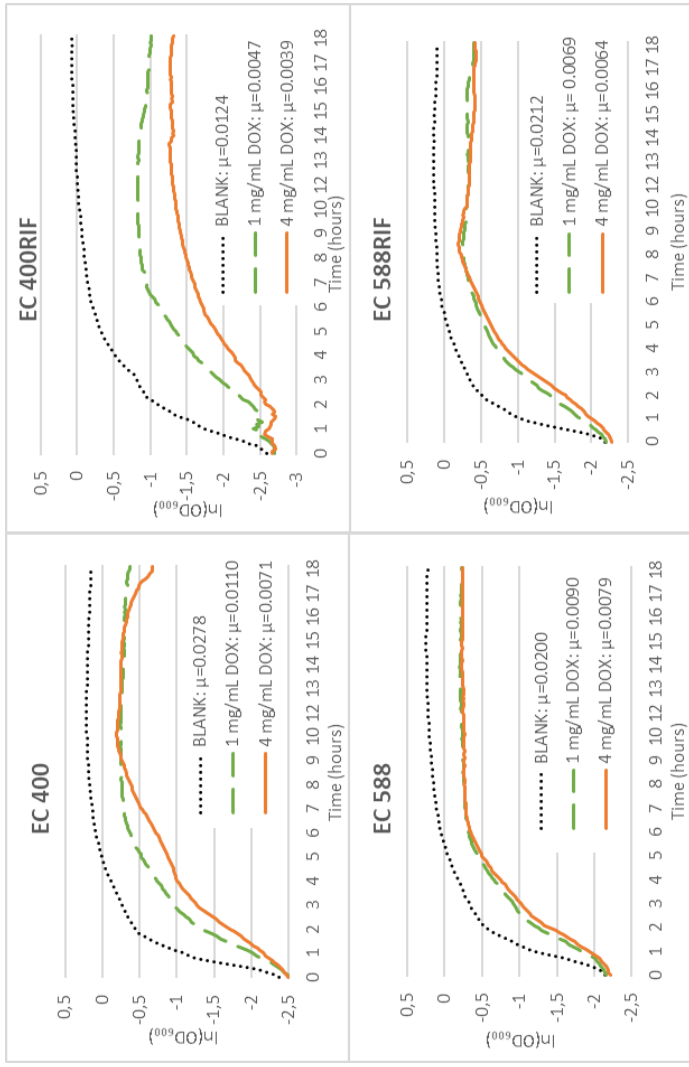


Figure 24. Growth curves of non-lactose fermenting mutants of doxycycline resistant *E. coli* EC 682, EC 202 and EC 292, susceptible *E. coli* EC 298, EC 400 and EC 588 and rifampicin resistant mutants EC 400RIF and EC 588RIF.

In each well of a 96-well plate, 100 µL of an overnight bacterial culture in Miller's LB broth (LB) of approximately 10^5 colony forming units (cfu)/mL was added to 100 µL of LB with a final concentration of 0, 1 or 4 mg/L DOX, respectively. The plate was incubated at 37°C in an automated microbiology growth curve analysis system (Infinite 200Pro, Tecan Group Ltd., Männedorf, Switzerland) during 18h and every 5 min OD_{600} was measured.



Each graph compares the growth of a strain in blank LB broth and LB with 1 and 4 mg/L doxycycline, incubated at 37°C during 18h (mean values of triplicates). The y-axis represents ln transformed OD_{600} values and the x-axis shows the incubation time (hours). The growth rates (μ, min^{-1}) were calculated manually according to Hall *et al.* and are given in the figure legends. The growth curves of the resistant strains EC 682, EC 202 and EC 292 represent the lactose negative mutants of these strains. Growth curves of the original lactose fermenting strains were also performed but are not shown. These growth curves did not differ from the growth curves of the lactose negative mutants.

4.2 GENETIC MAPPING OF TET(A)-CARRYING PLASMIDS

Replicon typing showed that plasmids pEC682, pEC202 and pEC292 belonged to the IncI1, IncFII, and the IncFII-FIB group, respectively. Plasmid sequencing of pEC682 revealed that the *tet*(A) gene (**Figure 25**) was flanked by *tet*R and located on a transposon (Tn7), together with mercury resistance genes. The co-transfer of four other resistance determinants together with the tetracycline resistance (**Table 8**) was confirmed by the presence of the corresponding resistance genes on pEC682: the bla_{TEM-1} gene encoding resistance to narrow-spectrum β-lactamases was located on a second transposon (Tn3) close to an integron class 1 that carried genes conferring resistance to sulphonamides (*sul*2), streptomycin (*str*A and *str*B) and trimethoprim (*dfhr*1) and a multidrug efflux pump coding gene (*emr*E). BLAST analysis showed that this plasmid shared nucleotide sequence identity with three other plasmids previously described from chicken caeca (pE17.16, accession number CP008733) (Brouwer *et al.* 2014), human faeces (FHI4, accession number LM995927) and pig faeces (PND11_107, accession number HQ114281) (Johnson *et al.* 2011).

Comparison of nucleotide sequences of pEC202 and pEC292 revealed that they shared the same backbone (**Figure 26**). The *tet*(A) gene of pEC292 was flanked by *tet*R and located on a Tn7 transposon that also carried genes conferring resistance to streptomycin, chloramphenicol and trimethoprim (*ant*1_1 and *ant*1_2, drug transporter of *bcr/Cfl*A subfamily, *dfr*A, respectively). This explains the co-transfer of these resistances (**Table 8**). Additionally, the transposon carried metal resistance coding genes and a multidrug efflux pump encoding gene. For plasmid pEC202, the direct environment of *tet*(A) was identical to that of pEC292. Both pEC202 and pEC292 shared their backbone sequence with a plasmid from a previously described human *E. coli* isolate (accession number KJ484634) (Wang *et al.* 2014).

All susceptible strains, except for EC 298 that carried an IncFIB plasmid, were negative for plasmids of the same Inc group as plasmids pEC682, pEC202 and pEC292.

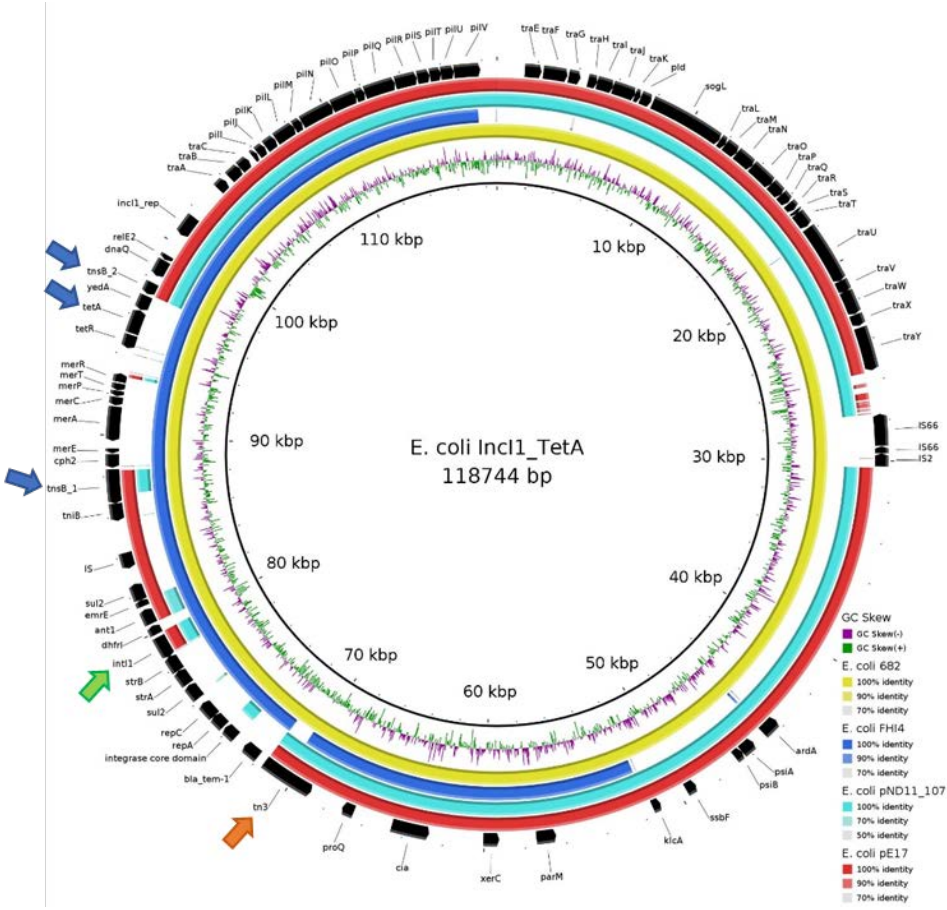


Figure 25. Partial nucleotide sequence of plasmid pEC682 (yellow) compared with sequences of plasmids FHI4 (dark blue, of human origin), pND11_107 (light blue, of porcine origin) and pE17 (red, of poultry origin). Relevant genes are indicated as black arrows. The *tet*(A) gene and the transposon were it is located on, are indicated with blue arrows. The transposon carrying the *bla_tem_1* gene is indicated with an orange arrow. The integrase gene of the integron is indicated with a green arrow.

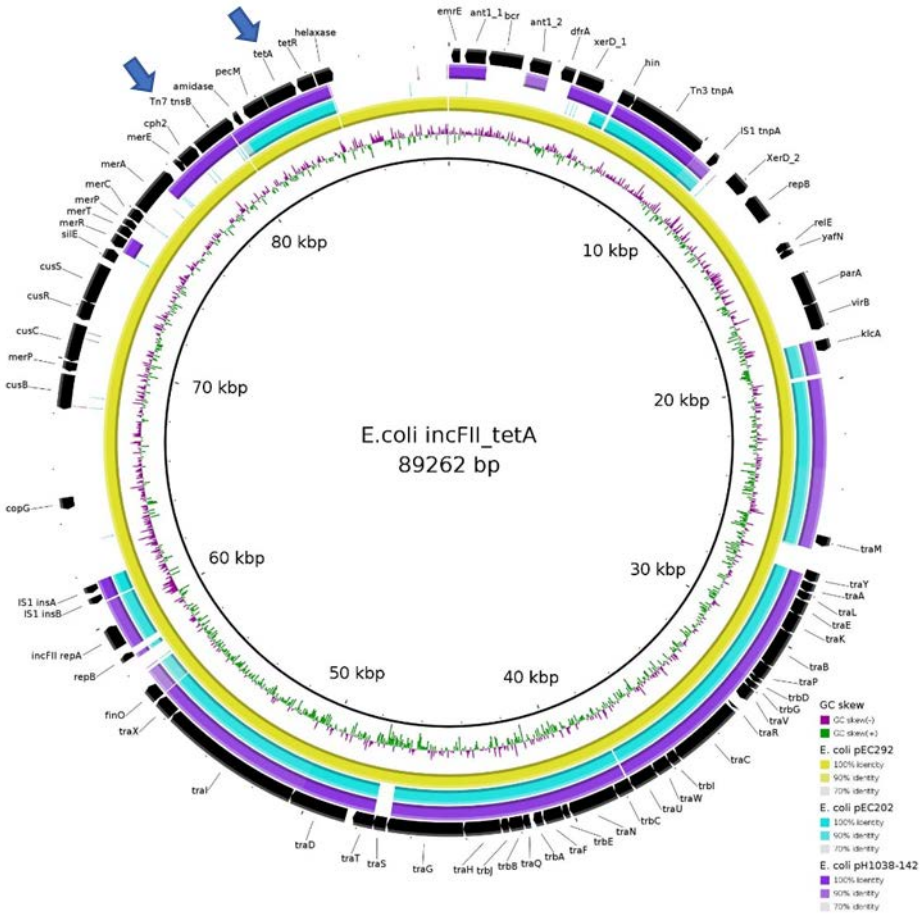


Figure 26. Partial nucleotide sequences of plasmid pEC292 (yellow) and pEC202 (light blue) compared with plasmid pH1038-142 of human origin (purple). Relevant genes are indicated with an arrow. Note: the gene denoted by 'bcr' encodes for a drug transporter of the bcr/CflA subfamily conferring resistance to chloramphenicol. The *tet*(A) gene and the transposon were it is located on, are indicated with blue arrows.

4.3 RESIDUAL DOX CONCENTRATIONS (1 AND 4 MG/L) CAN SELECT FOR DOX RESISTANT *E. COLI*

Figure 27 shows the ratios between DOX resistant strains EC 682, EC 202 and EC 292 and susceptible strains EC 298, EC 400, EC 400RIF and EC

588RIF during 48h of competition in LB with different concentrations of DOX. EC 400 and its RIF resistant mutant EC400RIF were included as different strains because of their difference in growth rate and MIC for DOX.

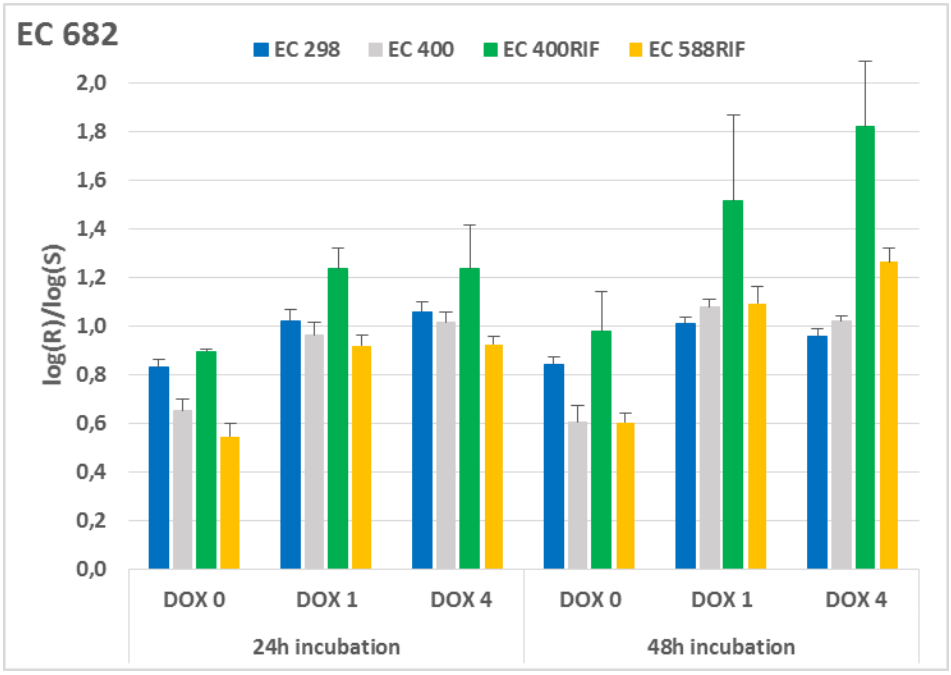
The selective effect on the resistant strain in the competition experiments is represented by the resistant/susceptible ratio (R/S). For every strain combination, statistical analysis revealed a significant higher R/S at 1 and 4 mg/l DOX when compared to the blank LB. In contrast, no significant difference in R/S was found between 1 and 4 mg/l DOX.

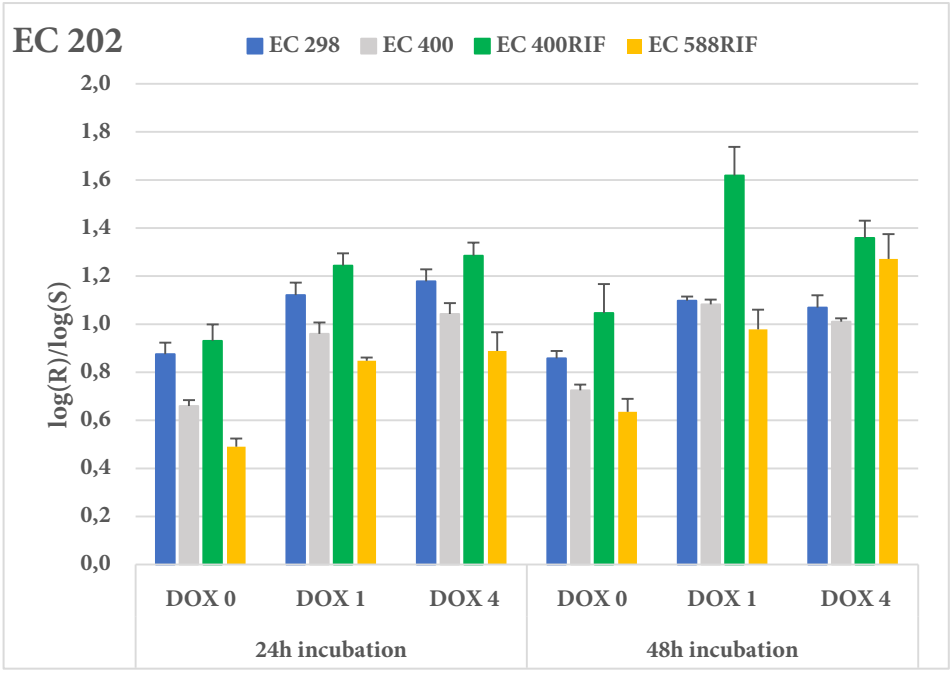
EC 298 in particular seemed to show a different behaviour compared to the other susceptible strains. Indeed, the R/S in DOX media for EC 298 looks slightly lower after 48h of incubation than after 24h, whereas the opposite occurred for the other susceptible strains.

Enumeration data of donor, recipient and transconjugant strains and data used for statistical analyses of R/S and transfer frequencies are given in **Table S2** (EC 298), **Table S3** (EC 400), **Table S4** (EC 400RIF) and **Table S5** (EC 588RIF).

The transfer frequencies of plasmids pEC682, pEC202 and pEC292 to recipient strains EC 400RIF and EC 588RIF under the experimental conditions are given in **Table 9**. The transfer frequency of pEC682 and pEC202 did not increase in LB with 1 or 4 mg/l DOX compared to the blank control. However, the transfer frequency of pEC292 was significantly higher at 1 mg/l DOX than at 0 ($p=0.009$) and 4 mg/l DOX ($p=0.001$) when EC 400RIF was used as recipient and significantly higher at 1 and 4 mg/l (both $p=0.02$) than in blank LB in case of recipient strain EC 588RIF.

LC-MS/MS analysis showed that the recovery of DOX in LB at concentrations of 1 and 4 mg/l ranged from 65% to 76% of the initial concentration after 48h of incubation at 37°C (**Table 10**).


EC 682
EC 298
EC 400
EC 400RIF
EC 588RIF
log(R)/log(S)
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
DOX 0
DOX 1
DOX 4
DOX 0
DOX 1
DOX 4
24h incubation
48h incubation


EC 202
EC 298
EC 400
EC 400RIF
EC 588RIF
log(R)/log(S)
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
DOX 0
DOX 1
DOX 4
DOX 0
DOX 1
DOX 4
24h incubation
48h incubation

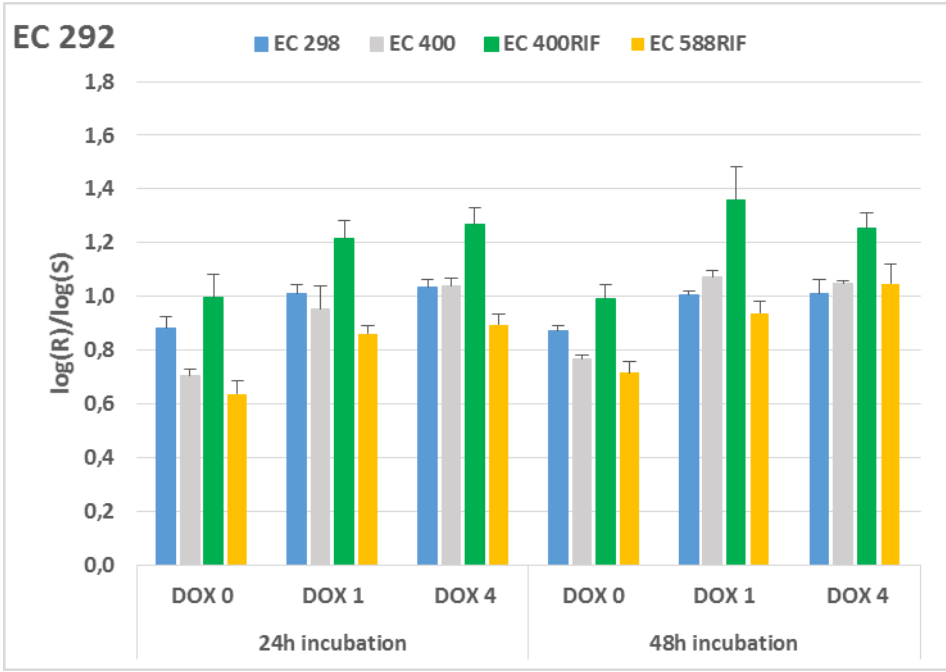


Figure 27. (previous page, this page) Results of competition experiments between doxycycline resistant strains EC682, EC202 and EC292 and susceptible recipient strains EC298 (blue), EC400 (gray), EC400RIF (green) and EC588RIF (yellow). The bars represent the ratio of logarithmic transformed counts of resistant bacteria (R, cfu/ml) to logarithmic transformed counts of susceptible bacteria (S, cfu/mL) (+ standard deviation), as indicated on the y-axis. In case of susceptible strains EC 400 and EC 298, R = total counts of donor (cfu/mL) and S = total counts of recipient (cfu/mL). In case of susceptible strains EC 400RIF and EC 588RIF, R = total counts of donor + tranconjugants (cfu/mL) and S = total counts of recipient – transconjugants (cfu/mL). Different concentrations of DOX in the medium (DOX 0 = 0 mg/l; DOX 1 = 1 mg/l; DOX 4 = 4 mg/l) and sampling time points (24h, 48h) are indicated on the x-axis. Significant differences are denoted in Additional files S1, S2, S3 and S4. **Note**: Statistical analysis was done using the log transformed ratios log(R/S) but the graph shows log(R)/log(S) to obtain a better image.

Table 9. Transfer frequency of resistance plasmids to susceptible *E. coli* EC 400RIF and EC 588RIF

		EC 400RIF		EC 588RIF	
		24h	48h	24h	48h
pEC682	Blank	1,58E-05	1,85E-03	1,57E-06	6,88E-05
	1 mg/l DOX	1,69E-05	1,30E-03	3,36E-06	1,19E-04
	4 mg/l DOX	1,01E-05	9,98E-04	7,35E-07	7,46E-04
pEC202	Blank	1,04E-05	1,73E-04	1,59E-05	3,43E-04
	1 mg/l DOX	8,27E-05	6,95E-04	6,08E-06	9,68E-04
	4 mg/l DOX	3,82E-05	9,17E-05	1,85E-07	1,51E-05
pEC292	Blank	3,21E-05	5,20E-04	5,04E-07	6,36E-06
	1 mg/l DOX	1,78E-04	2,22E-03	2,83E-05	9,24E-04
	4 mg/l DOX	5,88E-05	7,79E-05	1,97E-05	1,53E-02

Mean values of 4 replicates. Transfer frequency was defined as the number of transconjugants (CFU/ml) divided by the total number of recipients (CFU/ml) and was determined in three types of LB medium: Blank, 1 mg/l DOX and 4 mg/l DOX. Enumerations were performed after 24h and 48h of competition between donor and recipient strain. CFU: colony forming units; LB: Miller's LB broth; DOX: doxycycline

Table 10. Stability of DOX in LB broth (30 ml) with or without inoculation of strain EC 682 during 48 hours of incubation at 37°C on a horizontal shaker

Initial DOX concentration (mg/l)	Medium	Percentage of initial concentration after 23h	Percentage of initial concentration after 48h
1	LB	88	70
	LB + EC 682	82	65
4	LB	86	76
	LB + EC12 682	87	71

5 DISCUSSION

Characterization of the *tet*(A) carrying plasmids indicated that all three plasmids had well conserved backbones that circulate between *E. coli* strains from different hosts (human, poultry, pigs) (Brouwer *et al.* 2014, Johnson *et al.* 2011, Wang *et al.* 2014). In all three characterized resistance plasmids, *tet*(A) was located on a Tn*7* transposon together with several metal resistance genes. A transposon has the ability to transfer from the plasmid to another plasmid or chromosome in the same or another cell, and therefore can quickly spread resistance genes among bacteria (Salyers *et al.* 1995). Indeed, it has been shown that Tn*7* preferentially inserts into bacterial plasmids that can conjugate between cells (Wolkow *et al.* 1996). In case of pEC682, additional resistance genes were located on an integron, a genetic element that has the ability to capture resistance gene cassettes (Cambray *et al.* 2010). Consequently, although tetracyclines may not be the most critical or most frequently used group of antimicrobials for human medicine, the horizontal spread of these integron containing plasmids could be relevant because of co-transfer or acquisition of resistance genes other than *tet*(A).

None of the investigated conjugative plasmids were able to transfer to the susceptible strain EC 298. EC 298 carried a plasmid of the same Inc group as pEC292, which counteracts conjugation with EC 292 because plasmids of the same Inc group cannot be propagated within the same cell line (Carattoli *et al.* 2005). Conjugation with the other strains (EC 202 and EC 682) could possibly be counteracted by surface exclusion or a restriction system of strain EC 298 (Thomas *et al.* 2005).

Competition between the strains containing the *tetA*-carrying plasmids and susceptible strains at different residual DOX concentrations revealed one main observation: selection of the DOX resistant strain was in

all cases significantly higher in LB with 1 and 4 mg/L of DOX compared to the blank control. Surprisingly, no significant difference in selective effect was found between the two concentrations of DOX. This could partially be explained by the growth curve analysis of the susceptible strains (**Figure 24**). First, growth rates of the susceptible strains showed minor differences when comparing 1 and 4 mg/l of DOX. Second, all strains except EC 400RIF reached the same maximum OD value in both 1 and 4 mg/l of DOX. On the other hand, the rather high standard deviation (SD) values for the mean R/S (**Table S2—S5**), indicate that more replicates of the experiments might reveal more significant differences in selective effect between 1 and 4 mg/l of DOX, especially in case of EC 400RIF.

Not only the presence of DOX in the growth medium affected the competition results, also strain fitness played its role. First, the three resistant strains showed similar growth rates. As a result, no significant differences in R/S between these strains in competition with any of the susceptible strains were found. In contrast, the susceptible strains did show significant differences in R/S, except when comparing EC 400 and EC 588RIF. These differences are probably mainly caused by the (sometimes small) differences in growth rates (fitness) between the susceptible strains (**Figure 24**). Moreover, the differences in fitness between EC 400 and EC 400RIF and between EC 588 and EC 588RIF could possibly be assigned to the RIF mutation (Hughes *et al.* 2013). The fitness cost caused by the RIF mutation may also have affected the results of the standard MIC test with fixed incubation time, that showed different MIC values for the original strains and their RIF mutant, respectively.

Susceptible strain EC 298 in particular showed an interesting behaviour: the selective effect on any of the resistant strains in competition with EC 298 decreased with time. This observation could be caused by *de novo* resistance development with low fitness cost of EC 298 or a type of re-

sistance called 'adaptive resistance', a phenotype that can emerge through contact with subinhibitory concentrations of antibiotics (Andersson *et al.* 2014, Sandoval-Motta *et al.* 2016). Moreover, these hypotheses were supported by the growth curve of EC 298 (**Figure 24**) that reaches a platform after approximately 5-6 hours but increases again after 8-9 hours, indicating that the bacteria were adapting to the DOX supplemented environment.

Only the transfer frequency of pEC292 was increased due to the presence of 1 or 4 mg/l DOX. The lower transfer frequency to recipient EC 400RIF at 4 mg/l than at 1 mg/l could possibly be explained by the lower fitness of EC 400RIF at 4 mg/l than 1 mg/l DOX. Indeed, our experimental design implicates that the transfer frequency is likely a combined result of both plasmid transfer and enrichment of transconjugants (Lopatkin *et al.* 2016). Hence, EC 400RIF transconjugants were probably enriched significantly less at 4 mg/l of DOX than at 1 mg/l.

Interestingly, DOX appeared to be rather unstable under the experimental conditions: after 48h of incubation the recovery ranged between 65-76% (**Table 10**). This partial recovery was not translated into lower selective effects at 48h compared to 24h of incubation. In contrast, resistant/susceptible ratios were found to be significantly higher after 48h incubation time than 24h incubation time in all cases except for the experiments with EC 298. No previous reports about DOX stability in LB are available, but it has been shown that DOX is stable in deionized water at 37°C during at least 3 days (Honnorat-Benabbou *et al.* 2001). In contrast, hardness and pH of drinking water are main parameters determining stability of DOX in medicated drinking water. Moreover, the samples were protected from light during incubation, which rules out degradation due to light exposure. Therefore, it is more likely that the chemical properties of LB, e.g. the presence of divalent cations with subsequent binding of

DOX, caused the incomplete recovery but further investigation is needed to confirm this.

To our knowledge no previous studies on the selective pressure of residual concentrations of DOX are available in literature, except for one study that determined a Predicted No Effect Concentration (PNEC) for resistance selection of all common antimicrobials through a mathematical model (Bengtsson-Palme *et al.* 2016). According to this model the PNEC for DOX should be 0.002 mg/l, which means that the present results do not contradict the model.

In conclusion, this study showed that residual concentrations of DOX (1 and 4 mg/l), likely to be found in the porcine caecum and colon due to cross-contamination of feed, have the potential to enrich tetracycline resistant commensal *E. coli in vitro*. Consequently, cross-contamination of pig feed could be an important contributor to the spread of bacterial resistance (Andersson *et al.* 2014). Tetracycline resistant bacteria from the porcine microbiota can subsequently spread to the human microbiota through the consumption of pork or via the environment. Therefore, the presence of residual concentrations of DOX in the porcine microbiota may form a threat to human health, in particular because of co-transfer of important resistance genes in human medicine together with the tetracycline resistance genes. However, further research is needed to fully understand the long term effect of cross-contaminated feed in the complex environment of the intestinal microbiota.

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7 SUPPLEMENTARY INFORMATION

Table S2 — S5 (following pages).

Table S2. Competition experiment (type 1) between susceptible *E. coli* EC 298 and three tetracycline resistant *E. coli* strains (EC 682, EC 202, EC 292)

Number of Resistant Bacteria*		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
	Blank	6,00E+06	2,01E+07	3,51E+07	1,46E+07	1,89E+07	1,06E+07	nd	1,62E+07	3,26E+07	3,35E+07	2,74E+07	7,95E+06
EC 682	1 mg/L DOX	1,19E+08	3,44E+08	1,62E+08	1,71E+08	1,99E+08	8,62E+07	6,20E+07	3,21E+08	1,01E+08	2,28E+08	1,78E+08	1,03E+08
	4 mg/L DOX	5,45E+07	1,40E+08	1,19E+08	1,28E+08	1,10E+08	3,30E+07	2,19E+07	6,45E+07	4,50E+07	2,13E+07	3,82E+07	1,80E+07
	Blank	4,50E+06	8,84E+07	3,71E+07	2,40E+07	3,85E+07	3,10E+07	1,05E+07	4,49E+07	7,06E+07	5,53E+07	4,53E+07	2,21E+07
EC 202	1 mg/L DOX	7,15E+07	2,25E+08	8,75E+07	1,27E+08	1,28E+08	5,95E+07	1,93E+07	2,03E+07	1,19E+08	2,56E+07	4,59E+07	4,20E+07
	4 mg/L DOX	3,65E+07	2,15E+08	1,48E+08	1,35E+08	1,34E+08	6,36E+07	1,72E+07	8,25E+06	1,40E+07	1,66E+07	1,40E+07	3,53E+06
	Blank	1,05E+07	8,61E+07	1,57E+07	4,24E+07	3,87E+07	2,99E+07	9,73E+07	8,38E+07	7,76E+07	4,63E+07	7,62E+07	1,87E+07
EC 292	1 mg/L DOX	8,16E+07	3,09E+08	8,90E+07	8,80E+07	1,42E+08	9,63E+07	2,54E+08	1,97E+08	1,65E+08	1,08E+08	1,81E+08	5,26E+07
	4 mg/L DOX	6,11E+07	2,86E+08	7,65E+07	1,15E+08	1,34E+08	8,94E+07	3,89E+07	3,05E+07	4,80E+07	1,32E+08	6,24E+07	4,07E+07
Number of Susceptible Bacteria*		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
	Blank	4,50E+08	8,15E+08	4,17E+08	5,35E+08	5,54E+08	1,57E+08	3,20E+08	1,33E+09	6,60E+08	5,35E+08	7,10E+08	3,75E+08
EC 682	1 mg/L DOX	1,98E+08	6,55E+07	8,50E+07	1,62E+08	1,28E+08	5,42E+07	1,15E+08	2,77E+08	7,80E+07	1,13E+08	1,46E+08	7,70E+07
	4 mg/L DOX	6,50E+07	3,10E+07	2,55E+07	3,40E+07	3,89E+07	1,54E+07	2,85E+07	2,27E+08	2,86E+07	4,62E+07	8,24E+07	8,35E+07
	Blank	3,71E+08	6,20E+08	6,25E+08	2,45E+08	4,65E+08	1,64E+08	6,50E+08	9,30E+08	9,40E+08	8,50E+08	8,43E+08	1,16E+08
EC 202	1 mg/L DOX	1,36E+07	3,50E+07	2,90E+06	1,63E+07	1,69E+07	1,16E+07	5,65E+06	4,15E+06	2,45E+07	3,83E+06	9,53E+06	8,67E+06
	4 mg/L DOX	5,60E+06	1,60E+07	6,85E+06	3,00E+06	7,86E+06	4,90E+06	4,30E+06	8,75E+06	4,20E+06	2,15E+06	4,85E+06	2,41E+06
	Blank	3,65E+08	4,50E+08	3,55E+08	3,45E+08	3,79E+08	4,18E+07	4,55E+08	5,00E+08	4,35E+08	6,30E+08	5,05E+08	7,59E+07
EC 292	1 mg/L DOX	1,37E+08	1,08E+08	9,40E+07	1,19E+08	1,14E+08	1,55E+07	1,50E+08	1,58E+08	7,90E+07	1,09E+08	1,24E+08	3,18E+07
	4 mg/L DOX	5,75E+07	8,75E+07	7,35E+07	6,25E+07	7,03E+07	1,15E+07	2,31E+08	6,70E+07	7,95E+07	5,70E+07	1,09E+08	7,09E+07

Table S2 (continued)

LOG10 (Resistant/Susceptible)*		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
	Blank	-1,88	-1,61	-1,07	-1,57	-1,53	0,29	-3,10	-1,91	-1,31	-1,20	-1,88	0,75
EC 682	1 mg/L DOX*	-0,22	0,72	0,28	0,02	0,20	0,35	-0,27	0,06	0,11	0,31	0,05	0,21
	4 mg/L DOX*	-0,08	0,65	0,67	0,58	0,46	0,31	-0,11	-0,55	0,20	-0,34	-0,20	0,28
	Blank	-1,92	-0,85	-1,23	-1,01	-1,25	0,41	-1,79	-1,32	-1,12	-1,19	-1,35	0,26
EC 202	1 mg/L DOX*	0,72	0,81	1,48	0,89	0,97	0,30	0,53	0,69	0,68	0,82	0,68	0,10
	4 mg/L DOX*	0,81	1,13	1,33	1,65	1,23	0,31	0,60	-0,03	0,52	0,89	0,50	0,33
	Blank	-1,54	-0,72	-1,35	-0,91	-1,13	0,33	-0,67	-0,78	-0,75	-1,13	-0,83	0,18
EC 292	1 mg/L DOX*	-0,22	0,46	-0,02	-0,13	0,02	0,26	0,23	0,09	0,32	0,00	0,16	0,12
	4 mg/L DOX*	0,03	0,51	0,02	0,26	0,21	0,20	-0,77	-0,34	-0,22	0,36	-0,24	0,41

The table shows the results (+ mean values and SD of quadruplicates 1- to 4-) of enumerations (after 24h and 48h) of resistant and susceptible bacteria and the results that were used for statistical analysis, namely LOG10 (Resistant/Susceptible). *: resistant = number of donor bacteria, susceptible = total number of recipient bacteria. DOX concentrations that resulted in significant higher LOG10 (Resistant/Susceptible) values and thus have a significant higher selective effect than blank medium are denoted by an *.

Table S3. Competition experiment (type 1)between susceptible *E. coli* EC 400 and three tetracycline resistant *E. coli* strains (EC 682, EC 202, EC 292)

Number of Resistant Bacteria*		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
	Blank	4,17E+05	1,25E+06	1,35E+06	1,48E+05	7,91E+05	5,19E+05	9,17E+05	8,33E+03	1,50E+05	2,50E+05	3,31E+05	3,49E+05
EC 682	1 mg/L DOX	3,17E+07	2,33E+08	3,17E+07	1,25E+07	7,73E+07	9,04E+07	1,83E+08	3,13E+08	2,85E+08	1,85E+08	2,41E+08	5,86E+07
	4 mg/L DOX	1,41E+08	1,03E+08	6,75E+07	1,12E+08	1,06E+08	2,61E+07	2,21E+08	9,75E+07	9,58E+07	1,28E+08	1,35E+08	5,09E+07
	Blank	1,42E+06	1,50E+06	4,08E+05	6,42E+05	9,92E+05	4,75E+05	2,87E+06	3,25E+06	1,98E+06	8,17E+06	4,07E+06	2,41E+06
EC 202	1 mg/L DOX	2,75E+07	1,88E+08	2,08E+07	2,42E+07	6,50E+07	7,08E+07	9,50E+07	2,41E+08	1,49E+08	2,03E+08	1,72E+08	5,52E+07
	4 mg/L DOX	1,64E+08	1,44E+08	1,82E+08	2,53E+08	1,86E+08	4,12E+07	1,28E+08	5,67E+07	4,42E+07	3,75E+07	6,67E+07	3,63E+07
	Blank	2,00E+06	6,00E+06	1,34E+06	1,54E+06	2,72E+06	1,91E+06	6,98E+06	1,38E+07	6,50E+06	1,39E+07	1,03E+07	3,55E+06
EC 292	1 mg/L DOX	2,33E+07	1,63E+08	4,00E+07	1,50E+07	6,04E+07	6,01E+07	1,43E+08	3,79E+08	1,81E+08	2,03E+08	2,26E+08	9,09E+07
	4 mg/L DOX	1,91E+08	2,23E+08	2,22E+08	2,75E+08	2,28E+08	3,02E+07	3,06E+08	2,20E+08	1,83E+08	1,88E+08	2,24E+08	4,94E+07
Number of Susceptible Bacteria*		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
	Blank	1,04E+09	9,75E+08	9,58E+08	1,24E+09	1,05E+09	1,13E+08	1,20E+09	1,31E+09	1,52E+09	1,63E+09	1,41E+09	1,70E+08
EC 682	1 mg/L DOX	2,81E+08	2,72E+08	5,92E+07	6,67E+06	1,55E+08	1,23E+08	6,58E+07	8,17E+07	2,58E+07	6,33E+07	5,92E+07	2,05E+07
	4 mg/L DOX	1,58E+08	9,92E+07	1,92E+07	2,50E+07	7,52E+07	5,70E+07	1,83E+08	8,42E+07	3,50E+07	5,75E+07	9,00E+07	5,66E+07
	Blank	1,21E+09	1,38E+09	1,12E+09	1,24E+09	1,24E+09	9,26E+07	1,04E+09	1,42E+09	1,42E+09	1,42E+09	1,32E+09	1,62E+08
EC 202	1 mg/L DOX	2,35E+08	2,69E+08	1,92E+07	2,50E+07	1,37E+08	1,16E+08	4,00E+07	5,33E+07	2,50E+07	4,42E+07	4,06E+07	1,02E+07
	4 mg/L DOX	1,49E+08	1,18E+08	3,33E+07	4,33E+07	8,58E+07	4,89E+07	8,08E+07	4,75E+07	4,83E+07	4,50E+07	5,54E+07	1,47E+07
	Blank	1,21E+09	1,76E+09	1,13E+09	1,10E+09	1,30E+09	2,67E+08	1,56E+09	1,93E+09	1,50E+09	1,46E+09	1,61E+09	1,89E+08
EC 292	1 mg/L DOX	2,21E+08	2,93E+08	6,67E+06	3,25E+07	1,38E+08	1,22E+08	7,42E+07	6,42E+07	6,08E+07	5,08E+07	6,25E+07	8,33E+06
	4 mg/L DOX	1,38E+08	1,48E+08	7,25E+07	6,83E+07	1,07E+08	3,67E+07	1,33E+08	1,54E+08	8,67E+07	1,13E+08	1,22E+08	2,50E+07

Table S3 (continued)

LOG10 (Resistant/Susceptible)*		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
	Blank	-3,40	-2,89	-2,85	-3,93	-3,27	0,44	-3,12	-5,20	-4,00	-3,82	-3,59	0,75
EC 682	1 mg/L DOX*	-0,95	-0,07	-0,27	0,27	-0,25	0,45	0,44	0,58	1,04	0,47	0,71	0,24
	4 mg/L DOX*	-0,05	0,02	0,55	0,65	0,29	0,31	0,08	0,06	0,44	0,35	0,26	0,16
	Blank	-2,93	-2,96	-3,44	-3,29	-3,15	0,21	-2,56	-2,64	-2,85	-2,24	-2,52	0,22
EC 202	1 mg/L DOX*	-0,93	-0,16	0,04	-0,01	-0,27	0,39	0,38	0,65	0,78	0,66	0,64	0,15
	4 mg/L DOX*	0,04	0,09	0,74	0,77	0,41	0,34	0,20	0,08	-0,04	-0,08	0,05	0,11
	Blank	-2,78	-2,47	-2,93	-2,85	-2,76	0,18	-2,35	-2,15	-2,36	-2,02	-2,20	0,14
EC 292	1 mg/L DOX*	-0,98	-0,25	0,78	-0,34	-0,20	0,63	0,28	0,77	0,47	0,60	0,57	0,18
	4 mg/L DOX*	0,14	0,18	0,49	0,60	0,35	0,20	0,36	0,15	0,32	0,22	0,27	0,08

The table shows the results (+ mean values and SD of quadruplicates 1- to 4-) of enumerations (after 24h and 48h of incubation) of resistant and susceptible bacteria and the results that were used for statistical analysis, namely LOG10 (Resistant/Susceptible). *: resistant = number of donor bacteria, susceptible = total number of recipient bacteria. DOX concentrations that resulted in significant higher LOG10 (Resistant/Susceptible) values and thus have a significant higher selective effect than blank medium are denoted by an *.

Table S4. Competition experiment (type 2) between susceptible *E. coli* EC 400RIF and three tetracycline resistant *E. coli* strains (EC 682, EC 202, EC 292)

Number of Donor Bacteria		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
EC 682	Blank	2,13E+07	1,45E+07	2,63E+07	2,03E+07	2,06E+07	4,17E+06	5,69E+07	1,70E+08	1,58E+07	6,87E+06	6,24E+07	6,49E+07
	1 mg/L DOX	9,50E+07	1,11E+08	1,09E+08	3,12E+08	1,57E+08	8,96E+07	4,08E+08	3,75E+08	2,28E+08	2,24E+08	3,09E+08	8,34E+07
	4 mg/L DOX	2,70E+08	2,84E+08	3,41E+08	2,08E+08	2,76E+08	4,74E+07	3,84E+08	4,58E+08	3,40E+08	1,72E+08	3,38E+08	1,05E+08
EC 202	Blank	2,50E+07	1,01E+07	3,42E+06	1,39E+08	4,44E+07	5,53E+07	1,79E+08	2,54E+08	5,24E+07	2,11E+08	1,74E+08	7,51E+07
	1 mg/L DOX	9,00E+07	7,50E+07	9,00E+07	2,77E+08	1,33E+08	8,32E+07	4,16E+08	3,05E+08	1,99E+08	2,21E+08	2,85E+08	8,53E+07
	4 mg/L DOX	4,41E+08	5,18E+08	3,08E+08	3,43E+08	4,03E+08	8,23E+07	1,98E+08	2,11E+08	4,50E+08	1,67E+08	2,56E+08	1,13E+08
EC 292	Blank	2,83E+08	7,50E+07	5,17E+06	1,96E+08	1,40E+08	1,07E+08	9,50E+07	9,13E+07	3,61E+07	3,54E+08	1,44E+08	1,23E+08
	1 mg/L DOX	1,73E+08	1,29E+08	1,44E+08	3,18E+08	1,91E+08	7,49E+07	5,13E+08	4,80E+08	2,93E+08	3,21E+08	4,01E+08	9,60E+07
	4 mg/L DOX	4,86E+08	5,48E+08	5,06E+08	3,44E+08	4,71E+08	7,65E+07	6,86E+08	6,04E+08	6,63E+08	2,34E+08	5,47E+08	1,83E+08
Total number of recipient		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
EC 682	Blank	1,68E+08	6,75E+07	1,88E+08	1,61E+08	1,46E+08	4,63E+07	1,09E+08	2,46E+06	2,19E+08	2,45E+07	8,86E+07	8,49E+07
	1 mg/L DOX	5,59E+06	3,20E+06	6,22E+06	1,40E+06	4,10E+06	1,92E+06	2,53E+05	5,40E+04	1,29E+06	4,00E+03	4,01E+05	5,23E+05
	4 mg/L DOX	8,50E+05	1,25E+05	2,45E+07	8,57E+05	6,59E+06	1,04E+07	1,39E+05	1,57E+04	3,38E+04	2,73E+03	4,77E+04	5,37E+04
EC 202	Blank	1,88E+08	7,61E+07	1,95E+08	1,99E+08	1,65E+08	5,12E+07	1,20E+08	3,90E+06	1,28E+08	4,85E+07	7,51E+07	5,14E+07
	1 mg/L DOX	4,59E+06	1,94E+06	4,55E+06	2,53E+06	3,40E+06	1,19E+06	2,03E+05	1,11E+05	3,21E+05	3,47E+04	1,67E+05	1,07E+05
	4 mg/L DOX	2,96E+06	2,91E+06	2,83E+06	1,13E+07	4,99E+06	3,62E+06	1,96E+06	3,83E+05	1,97E+06	1,85E+06	1,54E+06	6,69E+05
EC 292	Blank	1,44E+08	7,38E+07	2,04E+08	1,95E+08	1,54E+08	5,17E+07	1,28E+08	9,76E+07	2,93E+08	1,79E+08	1,75E+08	7,45E+07
	1 mg/L DOX	1,68E+06	1,24E+06	2,98E+06	1,96E+07	6,37E+06	7,66E+06	2,37E+06	2,33E+05	1,89E+06	4,19E+06	2,17E+06	1,41E+06
	4 mg/L DOX	5,53E+06	3,33E+06	3,15E+06	1,62E+07	7,05E+06	5,37E+06	1,06E+07	3,61E+06	9,57E+06	1,43E+07	9,53E+06	3,85E+06

Table S4 (continued)

Number of Transconjugants		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
EC 682	Blank	1,99E+03	2,03E+03	1,14E+03	2,47E+03	1,91E+03	4,79E+02	1,70E+04	1,72E+04	2,18E+04	3,88E+03	1,50E+04	6,68E+03
	1 mg/L DOX	5,00E+01	0,00E+00	2,90E+02	1,67E+01	8,92E+01	1,17E+02	8,75E+01	3,75E+01	8,33E+00	1,67E+01	3,75E+01	3,08E+01
	4 mg/L DOX	2,50E+01	0,00E+00	3,00E+01	8,33E+00	1,58E+01	1,22E+01	6,25E+01	0,00E+00	1,67E+01	8,33E+00	2,19E+01	2,42E+01
EC 202	Blank	2,60E+03	7,50E+02	1,00E+03	2,52E+03	1,72E+03	8,47E+02	5,19E+03	2,01E+03	1,07E+04	2,32E+03	5,05E+03	3,48E+03
	1 mg/L DOX	2,25E+02	5,00E+01	8,80E+02	1,58E+02	3,28E+02	3,25E+02	3,38E+02	2,50E+01	2,25E+02	6,67E+00	1,49E+02	1,39E+02
	4 mg/L DOX	8,75E+01	1,25E+01	1,69E+02	6,67E+02	2,34E+02	2,56E+02	4,00E+02	1,25E+01	0,00E+00	2,40E+02	1,63E+02	1,67E+02
EC 292	Blank	6,13E+03	7,50E+02	1,03E+02	1,46E+04	5,39E+03	5,81E+03	1,18E+05	5,98E+04	1,08E+05	3,14E+04	7,94E+04	3,55E+04
	1 mg/L DOX	1,38E+02	1,88E+02	8,80E+02	3,56E+03	1,19E+03	1,40E+03	5,23E+03	1,26E+03	1,48E+03	1,89E+03	2,47E+03	1,61E+03
	4 mg/L DOX	1,38E+02	3,75E+01	9,50E+01	2,73E+03	7,51E+02	1,15E+03	6,25E+02	8,75E+01	6,67E+01	3,17E+03	9,88E+02	1,28E+03
LOG10 (Resistant/Susceptible)*		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
EC 682	Blank	-0,90	-0,67	-0,85	-0,90	-0,83	0,09	-0,28	1,84	-1,14	-0,55	-0,03	1,13
	1 mg/L DOX°	1,23	1,54	1,24	2,35	1,59	0,45	3,21	3,84	2,25	4,75	3,51	0,91
	4 mg/L DOX°	2,50	3,36	1,14	2,38	2,35	0,79	3,44	4,47	4,00	4,80	4,18	0,51
EC 202	Blank	-0,88	-0,88	-1,76	-0,16	-0,92	0,57	0,17	1,81	-0,39	0,64	0,56	0,81
	1 mg/L DOX°	1,29	1,59	1,30	2,04	1,55	0,30	3,31	3,44	2,79	3,80	3,34	0,36
	4 mg/L DOX°	2,17	2,25	2,04	1,48	1,99	0,30	2,00	2,74	2,36	1,95	2,27	0,32
EC 292	Blank	0,29	0,01	-1,60	0,00	-0,32	0,74	-0,13	-0,03	-0,91	0,30	-0,19	0,44
	1 mg/L DOX°	2,01	2,02	1,68	1,21	1,73	0,33	2,34	3,32	2,19	1,88	2,43	0,54
	4 mg/L DOX°	1,94	2,22	2,21	1,33	1,92	0,36	1,81	2,22	1,84	1,21	1,77	0,36

Table S5. Competition experiment (type 2) between susceptible *E. coli* EC 588RIF and three tetracycline resistant *E. coli* strains (EC 682, EC 202, EC 292)

Number of Donor bacteria		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
EC 682	Blank	1,00E+05	7,45E+03	1,51E+05	2,79E+05	1,34E+05	9,79E+04	1,17E+05	4,45E+04		5,65E+06	1,94E+06	2,63E+06
	1 mg/L DOX	3,76E+07	4,05E+07	3,96E+07	4,38E+07	4,04E+07	2,24E+06	1,13E+08	8,30E+07	5,03E+08	1,36E+08	2,09E+08	1,71E+08
	4 mg/L DOX	1,44E+07	4,95E+07	3,80E+07	4,18E+07	3,59E+07	1,31E+07	1,05E+08	4,60E+07	2,05E+08	1,53E+08	1,27E+08	5,86E+07
EC 202	Blank	5,00E+03	3,58E+04	5,25E+03	1,35E+05	4,53E+04	5,33E+04	1,30E+04	1,65E+04	6,87E+05	4,78E+04	1,91E+05	2,86E+05
	1 mg/L DOX	1,47E+07	2,86E+07	9,65E+06	5,40E+07	2,67E+07	1,72E+07	3,01E+07	2,22E+07	1,19E+08	5,75E+07	5,72E+07	3,80E+07
	4 mg/L DOX	9,75E+06	3,10E+07	2,52E+07	1,33E+08	4,96E+07	4,85E+07	7,60E+06	5,24E+07	2,68E+08	1,99E+07	8,70E+07	1,06E+08
EC 292	Blank	1,45E+05	4,34E+05	8,66E+04	4,96E+05	2,90E+05	1,77E+05	0,00E+00	1,24E+06	1,68E+07	1,04E+07	7,10E+06	6,88E+06
	1 mg/L DOX	1,61E+07	2,58E+07	2,00E+07	1,11E+08	4,31E+07	3,91E+07	5,20E+07	3,91E+07	2,10E+08	1,51E+08	1,13E+08	7,06E+07
	4 mg/L DOX	1,25E+07	3,24E+07	3,51E+07	1,74E+08	6,33E+07	6,42E+07	2,36E+07	4,00E+07	1,53E+08	1,29E+08	8,63E+07	5,55E+07
Total number of Recipient		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
EC 682	Blank	1,95E+08	1,32E+08	3,35E+08	1,11E+09	4,44E+08	3,94E+08	2,65E+08	6,93E+07	1,99E+09	9,60E+09	2,98E+09	3,89E+09
	1 mg/L DOX	1,68E+08	3,40E+07	1,63E+08	3,61E+08	1,81E+08	1,17E+08	1,50E+07	2,55E+07	4,60E+07	3,45E+08	1,08E+08	1,37E+08
	4 mg/L DOX	4,95E+07	6,20E+07	1,23E+08	2,85E+08	1,30E+08	9,36E+07	1,00E+06	2,32E+06	4,85E+06	6,65E+06	3,71E+06	2,19E+06
EC 202	Blank	7,50E+07	2,88E+08	7,25E+07	3,20E+09	9,08E+08	1,32E+09	1,70E+08	9,00E+07	1,54E+09	1,55E+09	8,36E+08	7,07E+08
	1 mg/L DOX	1,70E+08	2,54E+08	1,22E+08	1,25E+09	4,49E+08	4,65E+08	6,15E+07	1,51E+07	1,55E+07	4,30E+06	2,41E+07	2,21E+07
	4 mg/mL DOX	3,50E+06	2,47E+08	2,82E+08	1,44E+08	1,69E+08	1,08E+08	1,00E+06	5,20E+05	8,70E+05	7,30E+05	7,80E+05	1,78E+05
EC 292	Blank	7,50E+07	4,30E+07	1,07E+08	1,22E+09	3,61E+08	4,96E+08	1,60E+08	8,80E+07	1,47E+09	1,58E+10	4,37E+09	6,60E+09
	1 mg/L DOX	3,30E+08	2,10E+08	1,85E+08	5,18E+08	3,11E+08	1,32E+08	5,20E+07	2,44E+08	3,45E+08	1,12E+08	1,88E+08	1,14E+08
	4 mg/L DOX	5,45E+07	3,07E+08	6,95E+07	2,11E+08	1,60E+08	1,04E+08	2,36E+07	6,33E+06	8,35E+07	7,75E+08	2,22E+08	3,21E+08

Table S5 (continued)

Number of Transconjugants		1-24h	2-24h	3-24h	4-24h	Mean	SD	1-48h	2-48h	3-48h	4-48h	Mean	SD
EC 682	Blank	0,00E+00	2,55E+02	1,95E+02	4,21E+03	1,17E+03	1,76E+03	8,10E+03	1,63E+04	9,15E+03	4,52E+04	1,97E+04	1,50E+04
	1 mg/L DOX	5,00E+02	3,30E+02	2,00E+01	2,25E+02	2,69E+02	1,74E+02	4,70E+03	3,69E+03	3,55E+02	3,00E+03	2,94E+03	1,61E+03
	4 mg/L DOX	0,00E+00	1,80E+02	0,00E+00	1,00E+01	4,75E+01	7,66E+01	1,60E+03	3,04E+03	2,05E+02	2,35E+02	1,27E+03	1,17E+03
EC 202	Blank	3,00E+03	5,55E+03	2,70E+02	2,29E+03	2,78E+03	1,89E+03	0,00E+00	9,70E+04	4,54E+05	1,13E+03	1,38E+05	1,86E+05
	1 mg/L DOX	2,00E+03	2,76E+03	1,85E+02	1,40E+02	1,27E+03	1,14E+03	1,30E+03	5,75E+04	1,95E+02	7,00E+01	1,48E+04	2,47E+04
	4 mg/L DOX	0,00E+00	1,50E+01	5,50E+01	7,00E+01	3,50E+01	2,85E+01	0,00E+00	0,00E+00	5,00E+00	4,00E+01	1,13E+01	1,67E+01
EC 292	Blank	3,00E+01	6,50E+01	1,00E+01	1,50E+01	3,00E+01	2,15E+01	2,55E+03	8,10E+02	3,35E+02	1,77E+03	1,37E+03	8,55E+02
	1 mg/L DOX	2,08E+03	2,22E+04	1,00E+01	6,65E+02	6,24E+03	9,25E+03	2,75E+04	7,53E+05	1,96E+04	1,76E+03	2,00E+05	3,19E+05
	4 mg/L DOX	2,45E+02	2,19E+04	5,00E+00	6,30E+02	5,70E+03	9,36E+03	1,09E+04	3,83E+05	3,63E+03	6,00E+02	9,95E+04	1,64E+05
LOG10 (Resistant/Susceptible)*		**1-24h**	**2-24h**	**3-24h**	**4-24h**	**Mean**	**SD**	**1-48h**	**2-48h**	**3-48h**	**4-48h**	**Mean**	**SD**
EC 682	Blank	-3,29	-4,23	-3,34	-3,60	-3,62	0,37	-3,33	-3,06	nd	-3,23	-3,20	0,11
	1 mg/L DOX°	-0,65	0,08	-0,61	-0,92	-0,53	0,37	0,88	0,51	1,04	-0,40	0,51	0,56
	4 mg/L DOX°	-0,54	-0,10	-0,51	-0,83	-0,49	0,26	2,02	1,30	1,62	1,36	1,58	0,28
EC 202	Blank	-3,97	-3,84	-4,12	-4,37	-4,08	0,19	-4,12	-2,90	-3,13	-4,50	-3,66	0,67
	1 mg/L DOX°	-1,06	-0,95	-1,10	-1,36	-1,12	0,15	-0,31	0,17	0,89	1,13	0,47	0,57
	4 mg/L DOX°	0,44	-0,90	-1,05	-0,04	-0,39	0,62	0,88	2,00	2,49	1,43	1,70	0,60
EC 292	Blank	-2,71	-2,00	-3,09	-3,39	-2,80	0,52	-4,80	-1,85	-1,94	-3,18	-2,94	1,19
	1 mg/L DOX°	-1,31	-0,91	-0,97	-0,67	-0,96	0,23	0,00	-0,78	-0,22	0,13	-0,22	0,35
	4 mg/L DOX°	-0,64	-0,98	-0,30	-0,08	-0,50	0,34	0,00	0,83	0,26	-0,78	0,08	0,58

CHAPTER 5

Selection and transfer of an inci1-*tet*(a) plasmid of *escherichia coli* in an *ex vivo* model of the porcine caecum at doxycycline concentrations caused by cross-contaminated feed

Adapted from:

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1 ABSTRACT

The aim of this study was to investigate the effect of subtherapeutic intestinal DOX concentrations (4 and 1 mg/l), caused by cross-contamination of feed, on the enrichment of a DOX resistant commensal *E. coli* and its resistance plasmid in an *ex vivo* model of the porcine caecum.

A DOX resistant, *tet*(A) carrying, porcine commensal *E. coli* strain (EC 682) was cultivated for 6 days in the porcine caecum model under different conditions (0, 1 and 4 mg/l DOX). EC 682, other coliforms and anaerobic bacteria were enumerated daily. A selection of isolated DOX resistant coliforms (n=454) was characterized by rep-PCR clustering, PCR assays [Inc1 and *tet*(A)] and micro broth dilution susceptibility tests (Sensititre).

Both 1 and 4 mg/l DOX enriched medium had a significantly higher selective effect on EC 682 and other resistant coliforms than medium without DOX. Transconjugants of EC 682 were isolated more frequently in the presence of 1 and 4 mg/l DOX compared to medium without DOX.

Subtherapeutic intestinal DOX concentrations have the potential to select for DOX resistant *E. coli*, and promote the selection of transconjugants in a porcine caecum model.

Cross-contamination of feed with antimicrobials such as DOX likely promotes the spread of antimicrobial resistance. Therefore it is important to develop or fine-tune guidelines for the safe use of antimicrobials in animal feed and its storage.

2 INTRODUCTION

Antimicrobial resistance has traditionally been considered as a problem linked to the (mis)use of antimicrobials in human and veterinary medicine. During the last decade however, it has become clear that also low concentrations of antimicrobials may contribute to the selection and spread of antimicrobial resistance (Andersson *et al.* 2014), however the extent of this has not been quantified. Pig feed may become contaminated with antimicrobials through carry-over from medicated to non-medicated feed at the feed mill, during transport or at the farm (Filippitzi *et al.* 2016, Stolker *et al.* 2013). As the preferred administration methods for antimicrobials differ between countries, the main routes and levels of cross-contamination are also country-specific. Production of antimicrobial medicated feed at the feed mill has been banned in The Netherlands in 2011, consequently cross-contamination at the feed mill is ruled out in this country. The levels of cross-contamination at the feed mill can be highly variable. A wide range of antimicrobial concentrations have been found in a study concerning carry-over in 21 feed mills in The Netherlands (Stolker *et al.* 2013). Furthermore, it should be noted that the actual concentrations to which the pigs will finally be exposed to is also dependent on the half-life of the antimicrobial and other factors that influence the stability of the antimicrobial.

A mathematical model estimated that when 2% of the pig feed produced in a country per year is antimicrobial medicated feed, 5.5% (95% CI = 3.4%; 11.4%) of the total feed produced in a year is likely cross-contaminated with different concentrations of antimicrobials due to practices related to production, transport, storage and distribution of medicated feed (Filippitzi *et al.* 2016).

The concentrations of DOX, CTC or SDZ-TRIM in pigs' intestines, due to a 3% carry-over level in the feed, have been determined before in an *in vivo* study (Chapter 3). Based upon this information, it is possible to investigate the effect of the observed intestinal concentrations on the selection of resistant bacteria in the intestinal microbiota. The maximum concentration of DOX was approximately 4 mg/l in the porcine caecum and colon. Because the above mentioned study showed a high transfer rate of tetracyclines from feed to gut, it was decided to test the selective effect of the maximum observed concentration of DOX in caecum and colon (4 mg/l). As a consequence of a recent Belgian covenant (BFA and FASFC 2013), stating that carry-over levels of antimicrobials in pig feed should not exceed 1% of the recommended dose, the results of the *in vivo* study were also extrapolated to a 1% cross-contamination level (1 mg/l DOX).

The selective pressure of these two concentrations of DOX has recently been investigated using pure bacterial cultures (Chapter 4). These competition studies between DOX resistant and susceptible porcine commensal *Escherichia coli* strains showed that both 1 and 4 mg/l DOX supplemented medium select for the resistant strain compared to blank medium.

Taking into account the latter results, these low DOX concentrations might also exert a selective pressure on the porcine intestinal microbiota.

The low DOX concentrations may not only select for DOX resistant bacteria but may also promote the transfer of the tetracycline resistance genes. Moreover, other resistance genes carried by these bacteria could be co-selected and/or be co-transferred (Gullberg *et al.* 2014, Leverstein-van Hall *et al.* 2002).

The aim of the present study was thus to investigate the enrichment in the porcine caecal microbiota of a well characterized DOX resistant *E. coli* field strain, using an *ex vivo* model simulating the porcine caecum. This experiment allowed to observe two different mechanisms of resistance

spread: selection of the donor strain and transfer of its resistance plasmid, followed by selection of transconjugants. The resistant donor strain was characterized in a previous study (Chapter 4) and carried *tet*(A), encoding the efflux pump TetA, which is a concentration dependent resistance mechanism that confers resistance to tetracyclines (Moller *et al.* 2016). In addition, resistant coliforms other than the donor strain were characterized to determine whether the resistance plasmid of the donor strain was transferred to other strains.

3 MATERIAL AND METHODS

3.1 BACTERIAL STRAIN

EC 682 is a commensal *E. coli* strain that was isolated from pig faeces during a national Belgian antimicrobial resistance monitoring program (Hanon *et al.* 2015). EC 682 carries a mobile IncI1 plasmid (pEC682, EMBL accession number FNLQ01000000) conferring resistance to ampicillin, sulphonamides, streptomycin, tetracyclines and trimethoprim. Resistance to tetracyclines was encoded by the *tet*(A) gene located on the mobile plasmid pEC682 (Chapter 4). A non-lactose fermenting mutant of this strain was selected to be able to distinguish it from other (lactose fermenting) *E. coli* on MacConkey n°3 agar, on which this mutant forms white colonies (Smet *et al.* 2011). The non-lactose fermenting mutant showed the same MIC for DOX as the original strain, namely 16 mg/l. The *in vitro* growth rate of this strain (0.245 min^{-1}) was not affected by the presence of 1 mg/l DOX, whereas 4 mg/l DOX reduced the growth rate slightly by 0.0037 min^{-1}. The transfer frequency (ratio transconjugants/total recipients after 24h incubation) of pEC682 to two different recipient strains ($1{,}58.10^{-5}$ and $1{,}57.10^{-6}$, respectively) was not affected significantly by the presence of 1 mg/l DOX or 4 mg/l DOX (Chapter 4).

3.2 CÆCAL CULTURE CONDITIONS

The microbial ecosystem of the porcine caecum was simulated in an *ex vivo* model, described by (Messens *et al.* 2010). Briefly, the bacterial growth conditions of the porcine caecum were simulated in two parallel bioreactors, operated as continuous culture systems. The bioreactors both consisted of a BioFlo 110/115 unit (New Brunswick Scientific, Enfield, CT, USA) and a 1.3 l reactor vessel. At day 0, the reactor vessel was filled with 0.5 l of

nutritional medium (**Table 11**) and autoclaved (121°C, 30 min) and cooled down until 37°C. From that moment on, a constant temperature (37°C), pH (6.5) and agitation (150 rpm) were maintained and the headspace of the vessel was flushed constantly with a 80% nitrogen-20% carbon dioxide mixture at 20 ml/min to create anaerobic conditions.

Table 11. Composition of the nutritional medium

	g/l
Starch from corn (1)	5
Casein from bovine milk (1)	10
Casein hydrolysate acid (1)	0.5
Soybean oil (Carrefour sojaolie) (4)	1
L-cysteine hydrochloride anhydrous (1)	0.65
Pectin from citrus peel (1)	2.7
Alphacel (2)	13.8
Mucin from porcine stomach, type II (1)	5
Vitamin-mineral premix (5)	2.35
KH_2PO_4 (3)	0.93
$Na_2HPO_4 12H_2O$ (1)	1.12

(1) Sigma-Aldrich, Bornem, Belgium
(2) MP Biomedicals
(3) Merck, Overijse, Belgium
(4) N.V. Carrefour, Evere, Belgium
(5) Vitamex N.V., Drongen, Belgium
The medium was acidified to pH 2 with 4 ml/l HCl 37% (Merck)

Fermentation was started by adding 10 ml of pooled caecal content of 10 organic raised pigs that did not receive antimicrobials during rearing, and 1 ml of a 0.25 OD_{600} suspension of EC 682 (containing approximately 10^8 cells/ml). Immediately after inoculation, a sample was taken to determine the initial total count of EC 682 in the fermentation system. Subsequently, the reactor was operated in batch mode during 24h (day 0). Starting from

day 1, fresh medium of pH 2 (stored at 5°C in an autoclaved 13 l pyrex vessel) was added at a constant rate of approximately 1.8 ml/min and waste liquid and cells were removed at the same rate to obtain a constant working volume of 0.5 l. This corresponds with a retention time of approximately 4.6 h. A constant pH of 6.5 was maintained using a 3 mol/l NaOH solution. At day three, the nutritional medium was supplemented with 1 or 4 mg/l DOX (doxycycline hyclate, Fagron,Waregem, Belgium) and continuously administered until the end of the experiment, i.e. day nine. For each condition (0, 1 and 4 mg/l DOX), three reactor runs were conducted. Additionally, one run without EC 682 and without DOX was performed as a negative control experiment to determine whether IncI1 and *tet*(A) carrying plasmids were already present in the inoculum.

3.3 BACTERIAL POPULATION DYNAMICS

Tenfold serial dilutions of reactor content samples were plated daily in duplicate on MacConkey n°3 agar (MC) (Oxoid, Basingstoke, UK) with or without 8 mg/l DOX [= maximum cut-off value of DOX considering coliform bacteria according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing)] and incubated overnight at 37°C. Samples of the negative control run without EC 682 were plated on MC, MC + 4 mg/l DOX [= EUCAST ECOFF (epidemiological cut-off value) DOX for *E. coli*)] and MC + 8 mg/l DOX. EC 682 (white colonies) and other coliforms (red colonies) were counted on both MC with and without DOX. The number of susceptible coliforms was calculated by subtracting the resistant coliforms count from the total coliforms count. The same dilutions of the reactor content were also plated in duplicate on Reinforced Clostridial Agar (RCA, Oxoid) as a control to detect possible fluctuations in the culturable (facultative) anaerobic microbial population. The RCA plates

were incubated anaerobically during 48h at 37°C and the total number of colonies was counted.

3.4 ISOLATION OF DOX RESISTANT *E. COLI* AND IDENTIFICATION OF TRANSCONJUGANTS OF EC 682

Each day, 10 red colonies were randomly picked from MC + 8 mg/l DOX (resistant coliforms), further purified and stored at -80°C for further characterization. As the purpose of the negative control run was to detect IncI1 and *tet*(A) carrying isolates in the inoculum, a broad variety of resistant coliforms needed to be isolated. Therefore, also from the MC plates with 4 mg/l DOX, 10 colonies per day were isolated and stored. In this way, also resistant coliform species such as *E. coli* with a MIC value between 4 and 8 mg/l could be isolated. A representative collection (all isolates from day 3, 5, 7 and 9; in total 454) was selected for further characterization. These isolates were grown on RAPID' *E. coli* 2 agar (Bio-Rad, Temse, Belgium) to distinguish *E. coli* from other coliforms.

Genomic DNA of the 454 isolates was obtained using a boiling method. Briefly, one colony was suspended into 100 µl of ultra-pure water, heated during 10 min at 95°C in a warm water bath and finally centrifuged at 10,000 *g* during 2 min.

First, the 80 isolates originating from the negative control run without EC 682 were subjected to both IncI1 and *tet*(A) detecting PCR assays (Carattoli *et al.* 2005, Ng *et al.* 2001) to check whether coliforms carrying both *tet*(A) and the IncI1 replicon were already present in the inoculum. Next, the 374 isolates from the other runs were subjected to the IncI1 PCR assay to verify if they harboured plasmids with the IncI1 replicon. For isolates that carried the IncI1 replicon, the presence of *tet*(A) was also verified by PCR assay. Isolates that carried both the IncI1 replicon and the *tet*(A)

gene could be considered as possible transconjugants of donor strain EC 682.

All 454 isolates were then clustered into groups of indistinguishable or closely related isolates using rep-PCR with $(GTG)_5$ primers and under PCR conditions (Versalovic *et al.* 1991). The PCR mix consisted of 1× Red diamond buffer (Eurogentec, Seraing, Belgium), $(GTG)_5$ primer (100 pmol), 1.5 mM Mg_2Cl (Eurogentec), 1 U Red Diamond Taq DNA Polymerase (Eurogentec) and 0.2 mM of deoxynucleotide trisphosphates (GE Healthcare Europe, Munich, Germany) in a total reaction volume of 25 µl. This PCR mix was placed in a Gene Amp PCR System 9700 Gold (Applied Biosystems, Fostercity, CA, US). Amplicons were separated using capillary gel electrophoresis (QIAxcel Advanced System, QIAGEN, Hilden, Germany) with the QIAxcel DNA High Resolution Kit (QIAGEN) using method OM1200 with an additional 120-seconds separation time and the QX Alignment Marker (15 bp/3 kb, QIAGEN) added to each PCR product. The similarities between the obtained fingerprints were calculated using the Pearson correlation and clustered using UPMGA (1% curve smoothing) in BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium).

Subsequently, 16 IncI1 and *tet*(A) carrying isolates (at least one per rep-PCR cluster) and two isolates from the negative control run were selected for Sensititre micro broth dilution analysis with EUVSEC plates (TREK Diagnostic Systems, West Sussex, UK) according to EURL-AR (EURL-AR 2013) guidelines. The MIC's of the following panel of antimicrobials were determined: ampicillin, cefotaxime, ceftazidime, meropenem, nalidixic acid, ciprofloxacin, tetracycline, colistin, gentamicin, trimethoprim, sulphamethoxazole, chloramphenicol, azithromycin and tigecycline. Isolates were considered resistant or susceptible based on the cut-off values given in the EURL-AR guidelines (EURL-AR 2013). As such,

isolates showing resistances encoded by plasmid pEC682 (except for streptomycin, which is no longer included in the standard Sensititre plates) could be detected. The latter could then be considered as pEC682 carrying transconjugants, regarding that no such strains were isolated from the negative control run without EC 682.

3.5 STATISTICAL ANALYSIS

Significant changes as a response to the inclusion of DOX in the medium over a period of six days were analysed using a linear mixed effects model (Rpackage lme4, Bates *et al.* 2015), including 'medium' and 'time' as fixed factors. The reactor run number was considered as a random factor to include reactor variability in the model.

Statistical analysis was conducted on log transformed counts of EC 682, resistant and susceptible coliforms, total culturable anaerobes from reactor runs with 1 and 4 mg/l DOX compared to the runs with the blank medium. Only population sizes starting from day four until day nine were included for statistical analysis. P-values of significant results were calculated using ANOVA and the function lsmeans.

4 RESULTS

4.1 CAECAL BACTERIAL DYNAMICS AT 0, 1 AND 4 MG/L DOX

The EC 682 population size in the simulated porcine caecum was significantly higher ($p<0.001$) in reactors supplemented with 1 and 4 mg/l DOX (average increase of 1.20 ± 0.18 log_{10} CFU/ml and 1.19 ± 0.18 log_{10} CFU/ml respectively) compared to the blank controls (**Figure 28**).

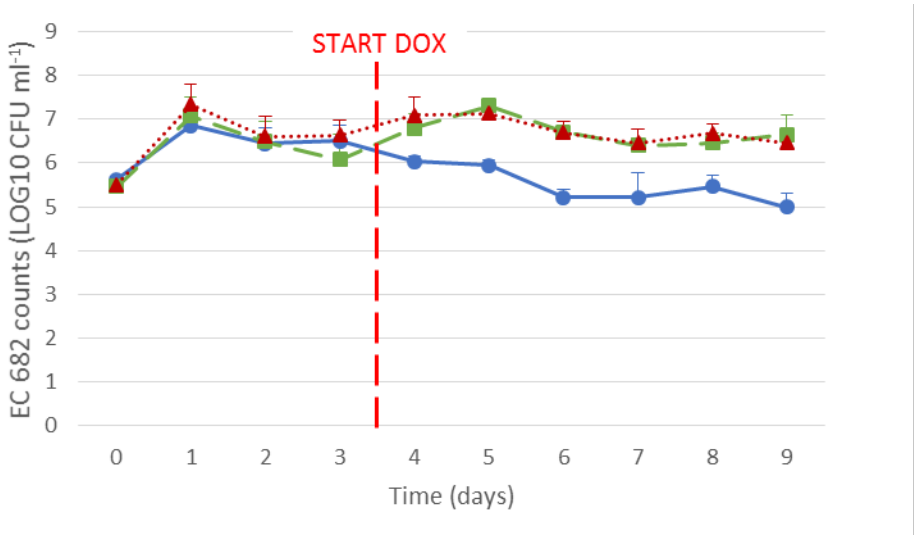

Figure 28. Enumeration of DOX resistant *E. coli* strain EC 682 in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg/l DOX. Strain EC 682 (white colonies) was enumerated daily in duplicate on MacConkey n°3 agar with 8 mg/l DOX (overnight incubation at 37°C) during 10 days. The population size of EC 682 is given in log transformed CFU/ml (mean values of three runs + SD) and plotted against time (days). The start of DOX administration is indicated with a red dashed line. The population size of the DOX resistant *E. coli* EC 682 after DOX administration (starting from day 4) was significantly higher in the presence of 1 and 4 mg/l DOX compared to blank medium. No significant difference in population size was observed between 1 and 4 mg/l DOX.

However, no difference in population sizes of EC 682 was observed between the two DOX concentrations. In addition, a significant effect ($p < 0.001$) of the factor 'time' on the population sizes of EC 682 was observed, since the EC 682 counts in blank medium decreased with time.

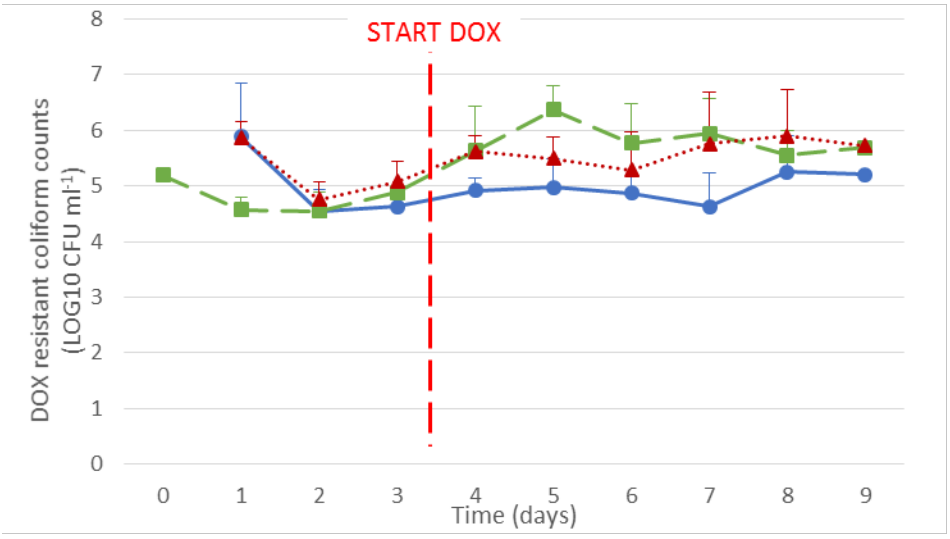

Figure 29. Enumeration of DOX resistant coliforms other than EC 682 in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg/l DOX. Resistant coliforms (red colonies) were enumerated daily in duplicate on MacConkey n°3 agar with 8 mg/l DOX (overnight incubation at 37°C) during 10 days. The population size of resistant coliforms is given in log transformed CFU/ml (mean values of three runs + SD) and plotted against time (days). The start of DOX administration is indicated with a red dashed line. Starting from day 4, the population size of the resistant coliforms was significantly higher in the presence of 1 mg/l DOX compared to the blank controls. No other significant differences were observed. **Note**: No growth could be observed at day 0 in the blank experiments and experiments with 4 mg/l DOX. The value given for day 0 of the experiments with 1 mg/l DOX, represents the count of only one of the three runs. The other two runs showed no growth at day 0.

In the experiments with 1 mg/l DOX, the resistant coliforms (**Figure 29**) showed a significantly ($p < 0.001$) higher population size (average increase of 1.17 ± 0.29 $\log_{10}$ CFU/ml) compared to the population size in the blank controls. No other significant differences in population sizes of resistant coliforms were observed. Also, no effect of time was observed for the population sizes of the resistant coliforms.

No significant differences in population sizes of the susceptible coliforms (**Figure 30**) were seen between any of the experiments, although the factor time did have a significant effect ($p = 0.016$) on these population sizes.

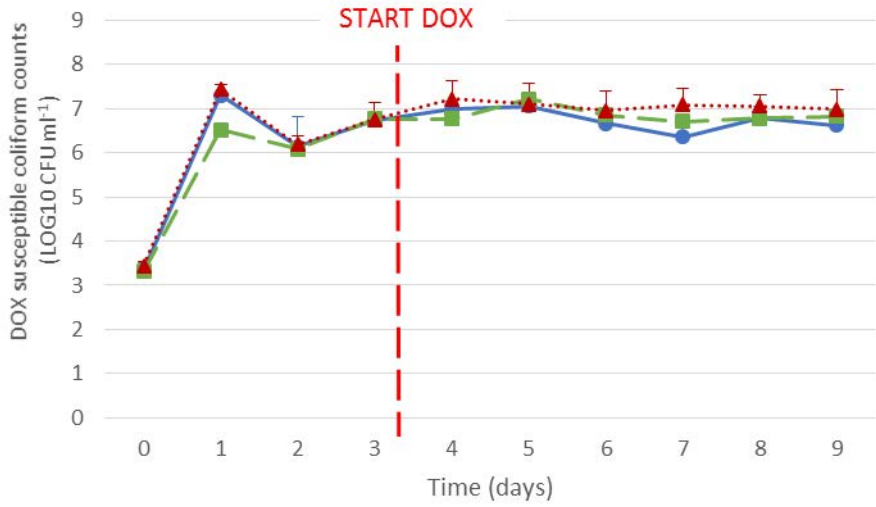

Figure 30. Enumeration of DOX susceptible coliforms in the simulated pig caecum containing 0 (blank ●), 1 (■) or 4 (▲) mg/l DOX. Total coliforms and resistant coliforms (red colonies) were enumerated daily during 10 days in duplicate on MacConkey n°3 agar without DOX and with 8 mg/l, respectively (overnight incubation at 37°C). The number of susceptible coliforms was calculated by subtracting the resistant coliform count from the total coliform count and is given in log transformed CFU/ml (mean values of three runs + SD) and plotted against time (days). The start of DOX administration is indicated with a red dashed line. No significant differences in population sizes of the susceptible coliforms were observed between any of the experiments.

Besides the coliforms, the population size of a more representative bacterial group of the microbiota was monitored by counting the total anaerobic bacteria on RCA (**Figure 31**). A significantly higher population size of these anaerobes was found in the experiments with 4 mg/l compared to those with 1 mg/l DOX (average increase of 0.42 ± 0.10 $\log_{10}$ CFU/ml, $p = 0.022$) and the blank controls (average increase of 0.28 ± 0.10 $\log_{10}$ CFU/ml, $p < 0.001$).

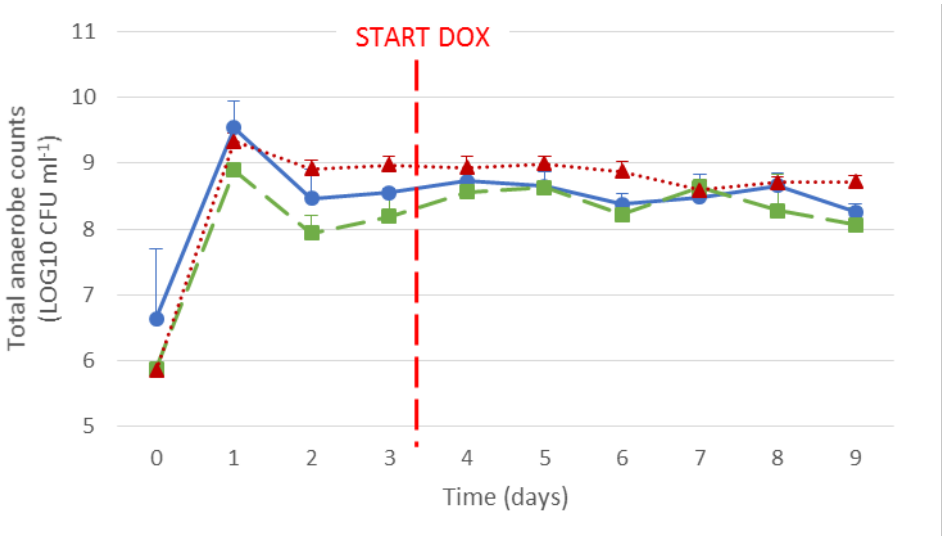

Figure 31. Enumeration of total anaerobes in the simulated pig caecum containing 0 (blank ●), 1 (■) or 4 (▲) mg/l DOX. Total anaerobes were enumerated daily in duplicate on Reinforced Clostridial Agar without DOX (48h anaerobic incubation at 37°C) during 10 days. The number of total anaerobes is given in log transformed CFU/ml (mean values of three runs + SD) and plotted against time (days). The start of DOX administration is indicated with a red dashed line. A significantly higher population size of anaerobes was observed in the experiments with 4 mg/l compared to those with 1 mg/l DOX and the blank controls. No other significant differences were seen.

4.2 DOX RESISTANT COLIFORMS AND PEC682 CARRYING TRANSCONJUGANTS

The donor strain EC 682 showed a rep-PCR pattern that was different from all DOX resistant coliforms that were isolated from the experiments. Of the 454 DOX resistant strains isolated from the blank experiments (n=132), the experiments with 1 mg/l DOX (n=123), those with 4 mg/l DOX (n=119) and the negative control experiment without EC 682 (n=80), 420 showed *E. coli* morphology on RAPID' *E. coli* 2 agar (**Table 12**). These 420 *E. coli* isolates were assigned to 41 different clusters by rep-PCR. One cluster (n° 8), including 127 *E. coli* isolates originating from the three different experimental runs, was remarkably larger than the others. Interestingly, no isolates from the negative control run were assigned to this large cluster. Other clusters comprised isolates originating from only one or two experiment type(s).

Table 12. Clustering of DOX resistant *E. coli* by rep-PCR and tet(A) and IncI1 carrying isolates

Run condition[a]	Number of isolates[b]	Clusters[c]	*tet*(A) and IncI1 carrying isolates (clusters)[d]
Blank	112	11	4 (2)
1 mg/l DOX	119	15	33 (5)
4 mg/l DOX	109	19	14 (4)

[a] For each condition, 3 runs were performed. Strains were isolated from all runs
[b] *E. coli* strains isolated on day 3 (just before DOX administration), 5, 7 and 9
[c] Total number of detected Rep-PCR clusters
[d] Total number of isolates with both IncI1 and *tet*(A) and between brackets the number of different clusters they were assigned to
Note: No *tet*(A) and IncI1 carrying isolates could be identified from the run without donor strain EC 682, the negative control experiment (80 isolates tested).

Fifty-one of the 420 *E. coli* isolates carried the IncI1 replicon, of which 4 originated from the blank runs, 33 from the runs with 1 mg/l DOX and 14 from the runs with 4 mg/l DOX. All 51 isolates also carried the *tet*(A) gene (**Table 12**) and were assigned to 11 clusters (**Table 13**). None of these 51 isolates could be assigned to the large cluster (n° 8).

The 34 isolates that showed different morphology on RAPID' *E. coli* 2 agar than *E. coli*, were assigned to 8 different clusters and did not carry the IncI1 replicon or *tet*(A) gene.

The 80 tetracycline resistant *E. coli* isolates originating from the negative control run without EC 682 were assigned to five different rep-clusters, of which four clusters also included isolates originating from the experimental runs. None of these 80 isolates were found to be both IncI1 and *tet*(A) positive, but 14 of them, belonging to 3 different clusters, did carry *tet*(A).

Sixteen IncI1 and *tet*(A) positive *E. coli* isolates (at least one isolate per cluster, see **Table 13**) were characterized with Sensititre. Eleven of these isolates showed the same resistance profile as EC 682 (**Table 13**). The other isolates from the experimental runs (n=5), only showed resistance against tetracycline. One of the two *tet*(A) carrying isolates from the negative control without EC 682 only showed resistance against tetracycline, the second isolate showed resistance to tetracycline and trimethoprim (**Table 13**).

Table 13. Phenotypic resistance profile of *tet*(A) and IncI1 carrying *E. coli* isolates

Run condition	Cluster n° (*a, b*)*	Strain n°	Phenotypic resistance profile
Blank	24 *(1, 1)*	135	tet
	25 *(3, 3)*	207	amp-smx-tet-tmp
		210	amp-smx-tet-tmp
1 mg/l DOX	5 *(6, 1)*	517	amp-smx-tet-tmp
	26 *(1, 1)*	304	amp-smx-tet-tmp
	36 *(8, 8)*	491	tet
		506	tet
	37 *(22, 22)*	324	amp-smx-tet-tmp
		385	amp-smx-tet-tmp
		403	amp-smx-tet-tmp
	38 *(1, 1)*	400	amp-smx-tet-tmp
4 mg/l DOX	22 *(5, 1)*	791	tet
	30 *(2, 1)*	747	tet
	32 *(7, 7)*	589	amp-smx-tet-tmp
	39 *(5, 5)*	728	amp-smx-tet-tmp
		744	amp-smx-tet-tmp
Blank without EC 682	5 *(6, 1)*[§]	1011	tet-tmp
	28 *(9, 0)*[†]	960	tet

amp, ampicillin; smx, sulphamethoxazole; tet, tetracycline; tmp, trimethoprim

* a: total number of isolates assigned to this cluster; b: total number of both IncI1 and *tet*(A) positive isolates in this cluster

[§] Isolate n° 1011 belonged to a cluster with five *tet*(A) positive isolates originating from the negative control run and one IncI1 and *tet*(A) positive isolate originating from an experiment with 1 mg/l DOX.

[†] Isolate n° 960 belonged to a cluster with eight *tet*(A) positive isolates and one *tet*(A) negative isolate, all originating from the negative control run.

5 DISCUSSION

Resistance selection in the pig microbiota caused by cross-contamination of feed with antimicrobials is worrisome, especially since it is assumed that resistant bacteria can be persistent and are thus not necessarily outcompeted by susceptible bacteria when antimicrobial selective pressure is withdrawn (Andersson *et al.* 2011). It has also been stated that, theoretically, sub-MIC selected resistant mutants of bacteria would be more stable in bacterial populations than those selected at high antimicrobial concentrations because of the lower fitness cost (Sandegren 2014). Therefore, the aim of this study was to investigate the selective effect of intestinal DOX concentrations due to cross-contamination of feed on the porcine microbiota.

The enumerations of DOX resistant *E. coli* EC 682 in the simulated porcine caecum clearly showed that both 1 and 4 mg/l of DOX have a selective effect on this strain. The population size of the phenotypically resistant EC 682 was maintained or increased slightly in the DOX supplemented media, while in the blank medium this population size decreased. Presumably, EC 682 was not able to maintain its population size in this latter medium due to the lack of selective advantage compared to the susceptible microbial population present in the reactor. In addition, EC 682 might have started eliminating its resistance plasmid (pEC682) in the absence of antimicrobial pressure because of the cost of fitness to replicate (Sherratt 1982), although we were not able to demonstrate this in this type of experiment. Furthermore, similar trends were observed in a previous study with a cefotaxime resistant *E. coli* strain in bioreactor experiments simulating the human caecum and ascending colon (Smet *et al.* 2011). Interestingly, no significant difference in selective effect between 1 and 4 mg/l was found. This finding could possibly be explained by the tetracycline resistance mechanism of EC 682, i.e. the TetA efflux pump and regulation

protein TetR, as a recent study showed that TetA producing *E. coli* exhibit a prolonged generation time with increasing tetracycline concentrations (Moller *et al.* 2016). Consequently, it is likely that the fitness of EC 682 was affected more in the medium with 4 mg/l compared to 1 mg/l DOX, thus neutralizing the potential higher selective effect of 4 mg/l DOX.

As EC 682 was not the only tetracycline resistant coliform present in the microbial population, the counts of coliforms were also investigated in general, and more specific to see whether transconjugants were arising. The resistant coliform counts were also affected by DOX supplementation (**Figure 29**), although statistical analysis only confirmed a selective effect in medium with 1 mg/l DOX. The coliform population comprises many different species, which likely harbour different types of resistance mechanisms concomitant with different fitness costs, which can explain variable selective effects depending on the DOX concentration (Vogwill *et al.* 2015). The prolonged generation time with increasing tetracycline concentrations of bacteria using an efflux pump as resistance mechanism could, in this case, also explain the lower selective effect of 4 mg/l DOX compared to 1 mg/l DOX (Moller *et al.* 2016).

Probably, the enrichment of the EC 682 population and other resistant coliforms did not affect the size of the susceptible coliform population because they represent a small minority in the total microbiota present in the bioreactor. Indeed, over 90% of the bacteria in the porcine caecum belong to the phyla Firmicutes and Bacteroidetes, whereas *E. coli* have been reported to represent between 0.72% and 4.8% of the microbiome (Leser *et al.* 2002, Yang *et al.* 2016).

The diversity of anaerobes that are culturable on RCA (i.a. Clostridia, Lactobacilli) presumably masks the effect of DOX supplementation on the anaerobic population. Different species can show different growth rates and can harbour different resistance mechanisms, which can each affect

the bacterial fitness differently (Vogwill *et al.* 2015). In general, the populations of anaerobes seemed to maintain more or less the size that was established before the start of DOX supplementation.

Regardless of the mechanism of selection, the characterization of the resistant coliforms showed that more pEC682 carrying *E. coli* could be isolated from the experiments with DOX supplementation compared to the blank experiment **(Table 13).** In other words, not only EC 682 itself but also its resistance plasmid pEC682, conferring resistance to five different antimicrobials, was enriched more in the experiments with DOX supplemented medium compared to the experiments with blank medium. It should be noted though that this was not a quantitative study, as this would require at least a systematical characterization of all isolates growing on one agar plate. Consequently, it was not possible to confirm the observed trends statistically. Although the clustering seemed to show a slightly larger variety of transconjugants in the experiments with DOX supplemented media, it is unclear whether the positive selection of the plasmid was caused by a higher plasmid transfer frequency, or by enrichment of transconjugants. This is only one of the various confounding factors that complicate the interpretation of plasmid transfer frequency, which make that conjugation dynamics under antimicrobial selective pressure are to date poorly understood (Lopatkin *et al.* 2016).

Our results are in line with previous *in vitro* competition experiments between susceptible commensal *E. coli* strains and EC 682 and two other *tet*(A) carrying commensal *E. coli*, where similar selective effects of 1 and 4 mg/l DOX with the same statistical significance were observed (Chapter 4). In addition, different studies confirm our finding that resistance genes conferring resistance to other antimicrobials than the one(s) administered can be co-selected (Agga *et al.* 2015, Leverstein-van Hall *et al.* 2002, Looft *et al.* 2012). The selection of tetracycline genes on multidrug resistance

plasmids obviously contributes to a widespread dissemination of multi-drug resistant enteric bacteria.

In conclusion, caecal concentrations of DOX (1 and 4 mg/l) caused by a 1% and 3% carry-over level of DOX in pig feed, have the potential to enrich *tet*(A) carrying *E. coli* in the porcine caecum. Since this study revealed that 4 mg/l DOX does not necessarily have a higher selective effect than 1 mg/l DOX, and previous observations indicate that very low antimicrobial concentrations (ng/ml) can select for persistent (*de novo*) resistance (Andersson *et al.* 2014, Gullberg *et al.* 2014, Gullberg *et al.* 2011), questions could be raised about the relevance of current maximum levels of cross-contamination of feed with respect to resistance selection. However, the type of antimicrobial and associated resistance mechanisms may strongly influence the extent to which selection of resistant bacteria occurs. Therefore, additional research is needed to elucidate quantitative differences in selective effect of different contamination levels of antimicrobials used in medicated pig feed, to be able to optimize legal limits for cross-contamination levels.

CHAPTER 6

General discussion

1 CROSS-CONTAMINATION OF PIG FEED AS A DRIVER FOR ANTIMICROBIAL RESISTANCE SELECTION

The continuing worldwide spread of antimicrobial resistance in both commensal and pathogenic bacteria is worrisome. The research results on doxycycline, described in this doctoral thesis, indicate that cross-contamination of pig feed with antimicrobials is a contributor to this phenomenon. It is however difficult to quantify this contribution as different factors may contribute: (1) the type and concentration of antimicrobial present in the feed, (2) the pharmacokinetic properties of the antimicrobial driving the intestinal concentrations of the drug and (3) the properties of the intestinal microbiota (MIC, resistance mechanisms, bacterial fitness, etc.). The main findings and results obtained in this doctoral thesis are shown in **Figure 32**.

1.1 ANTIMICROBIALS IN CROSS-CONTAMINATED FEED

The type and concentration of the antimicrobial that is present in the cross-contaminated feed is the first factor that will determine the effect on the intestinal microbiota. Unfortunately, only a minimal number of published studies investigated which type and concentrations of antimicrobials can be found in cross-contaminated feed. Only one study described quantitative data on cross-contamination at the feed mill (Stolker *et al.* 2013), whereas another study (Putier 2010) investigated cross-contamination during feed transport with trucks. No data, however, have been published regarding carry-over of antimicrobials at the farm. The study by Stolker and colleagues (2013) showed that cross-contaminated feed batches can contain a wide range of antimicrobial concentrations (with sometimes

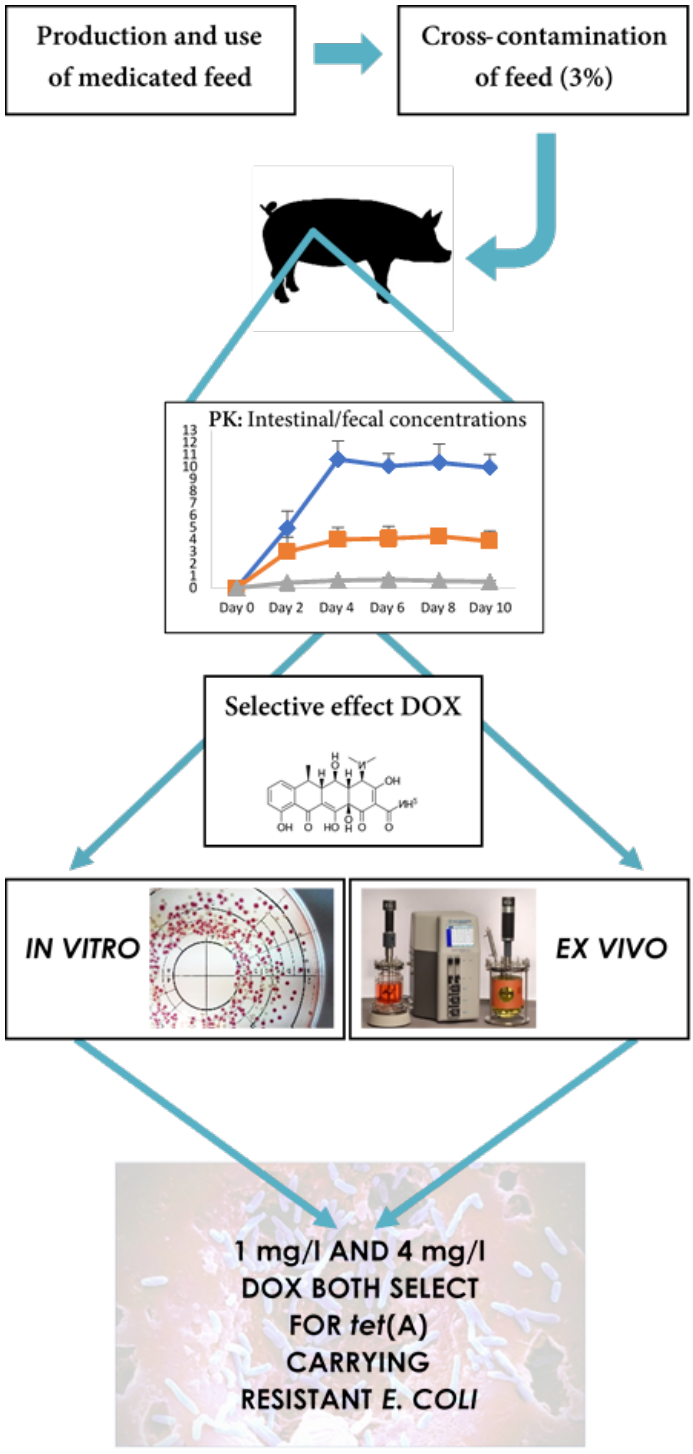

Figure 32. Schematic overview of the main results obtained in this doctoral thesis

even more than one type of antimicrobial), which impedes a quantitative estimation of the consequences of cross-contaminated feed (Stolker *et al.* 2013). The route of antimicrobial carry-over influences the final antimicrobial concentration in the cross-contaminated feed. In general, the level of contamination is expected to be higher when carry-over has taken place at the feed mill compared to carry-over at transport or farm level (Filippitzi *et al.* 2016). The Belgian Feed Association (BFA) estimated that the carry-over level at the feed mill reaches on average 2% when a main mixer is used, whereas it would stay below 1% when mixing at the end of the feed line is applied. Subsequently, a covenant with the Belgian Federal Agency for the Safety of the Food Chain (FASFC) was signed, which banned the use of main mixers in Belgian feed mills starting from Jan 2014.

Moreover, guidelines stating that carry-over of antimicrobials should not exceed 1% of the minimal allowed dose, were established (BFA and FASFC 2013).

The question remains if the residual antimicrobial quantities in feed resulting from cross-contamination remain stable until the feed is consumed. Manufacturers of premix formulations claim that these formulations are stable under normal conditions of use. This has been confirmed in a peer reviewed report concerning therapeutic doses of a sulfadimidine premix (Bernabeu *et al.* 2000), and for oxytetracycline when applied in a pellet feed (Counotte *et al.* 1984). Some studies have shown that tetracycline residues can be detected, and even accumulate, in organic matter such as farm dust and dried pig manure (Hamscher *et al.* 2003, Hamscher *et al.* 2002), which may also point towards good stability in feed. Similarly, sulphonamides could also be affected by UV exposure, although it has been reported for tetracycline-HCl that light exposure doesn't cause degradation when in dry state (Wu *et al.* 2005).

In short, there exists an important lack of data regarding exposure of animals to cross-contaminated feed. Quantitative data on carry-over levels (especially at the level of the transport, storage and distribution at the farm), as well as information on the destination of the cross-contaminated feed and the stability of the antimicrobial residues are very scarce. The mathematical model developed by Filippitzi and co-authors, that estimates the percentages of cross-contaminated feed produced in a certain country per year, could be refined substantially in case quantitative data would become available.

1.2 DISPOSITION AND PHARMACOKINETICS OF THE ANTIMICROBIAL DRIVING INTESTINAL CONCENTRATIONS

The disposition and pharmacokinetic properties of the antimicrobial present is another very important factor that contributes to the impact of cross-contaminated feed on resistance selection. The antimicrobial exposure of the intestinal microbiota after oral administration can be affected by the extent of drug absorption (i.e. oral bioavailability), the intestinal site of drug absorption, the extent of drug secretion from the systemic circulation into the gut and the proportion of drug that remains active in the gastrointestinal tract (i.e. unmetabolized and unbound). These properties are characteristic for each type of antimicrobial. In our first study (Chapter 3), we found a correlation between the oral bioavailability and the intestinal concentrations of the tested antimicrobials (doxycycline, chlortetracycline and sulphadiazine-trimethoprim). This means that transfer of the antimicrobial from feed to gut was high to very high in case of the tetracyclines, which have generally low oral bioavailability's in pigs (doxycycline 21-50% and chlortetracycline 6-18%), whereas sulphadiazine and trimethoprim (with a high oral bioavailability, sulphadiazine 85-90% and trimethoprim 73-92%) showed a low transfer from feed to gut. However, this correlation

of intestinal concentrations should be carefully interpreted, as other (unknown) pharmacokinetic properties might also influence intestinal concentrations. Furthermore the results should certainly not be extrapolated to other antimicrobials because each drug has its own specific properties. One of these typical properties of tetracyclines is enterohepatic circulation, which means that they are reabsorbed from the intestines and re-enter the systemic circulation via the liver (del Castillo 2013). This might have contributed to the high intestinal concentrations found in our study, and might imply a longer exposure of the microbiota. Although it seems logic that oral administration implies the highest risk for resistance selection in the intestinal microbiota, other administration routes should not be underestimated. A recent study by our group showed that sulphadiazine undergoes intestinal secretion to the gut lumen of pigs, independent of the administration route (oral and parenteral), although a renal excretion route has always been assumed for this drug. This causes an accumulation of sulphadiazine towards the distal parts of the gastrointestinal tract. Remarkably, trimethoprim did not show such an accumulation, pointing towards molecule-dependent mechanisms. In particular, ion-trapping in the gut lumen could explain the accumulation of sulphadiazine (De Smet *et al.* 2017). Accumulations were also observed in our study: maximum mean concentrations of sulphadiazine (0.67 mg/kg) were found in the distal colon ascendens and for both doxycycline and chlortetracycline in the colon descendens (doxycycline 3.81 mg/kg; chlortetracycline 10.08 mg/kg). No conclusions could be made for trimethoprim as all values except two were below the detection limit. We also observed accumulation towards the end of the gastrointestinal tract for doxycycline and chlortetracycline. This might partly be due to intestinal secretion via P-glycoproteins and biliary excretion, although tetracyclines are also reabsorbed from the intestines

(del Castillo 2013). The accumulation may also (partly) be due to the progressive water absorption from the intestinal content towards the end of the intestinal tract, as the antimicrobials were administered orally in our study.

Taking into account this important role of pharmacokinetics, a general limit for carry-over levels, e.g. the 1% proposed by the EU, can result in completely different intestinal concentrations for different antimicrobial drugs. As such, the different antimicrobials used in feed medication (amoxicillin, oxytetracycline, colistin, lincomycin, tilmicosin, tylosin, apramycin, spectinomycin, tiamulin and valnemulin) should be tested individually. For example, the frequently applied antimicrobial amoxicillin, has an oral bioavailability in pigs that ranges between 22 and 33% and is mainly excreted renally (Agerso *et al.* 1998, Reyns *et al.* 2007). Intestinal concentrations after oral administration of amoxicillin might thus be rather high, as was found for the tetracyclines. On the other hand, it has been shown that penicillins are unstable in faeces matrix (Berendsen *et al.* 2015).

1.3 THE INTESTINAL MICROBIOTA, RESISTANCE MECHANISMS AND THEIR GENETIC ENVIRONMENT

Taking into account the current knowledge on resistance selection and transfer at sub-MIC antimicrobial concentrations, there is a high probability that the gut ecosystems of animals and humans are important hot spots of resistance selection and transfer.

Making a link between antimicrobial use in pig production and antimicrobial-resistant bacterial infections in the human population is rather challenging, although some studies have demonstrated such a link. These studies however mainly focus on zoonotic bacteria and their resistance, rather than on the transfer of resistance to non-zoonotic human pathogens (Lu *et al.* 2002, Maguire *et al.* 1993, Molbak *et al.* 1999).

It is clear that resistance genes can be very persistent in the intestinal microbiota, even in long term absence of antimicrobials (Gerzova *et al.* 2015, Gumpert *et al.* 2017, Jakobsson *et al.* 2010). On the other hand, reductions in the use of antimicrobials can reduce the load of resistant bacteria successfully, as was shown in Belgian and Dutch monitoring reports in livestock (Callens *et al.* 2017, Dorado-Garcia *et al.* 2016, Hanon *et al.* 2015). Nevertheless, medication remains necessary in justified cases so the need to investigate resistance selection in the porcine intestinal microbiota remains actual.

The mammalian gut harbours approximately 500-1,000 species of bacteria which are present in large numbers, e.g. no less than 10^{11} bacteria per gram human colon content. The majority of these bacteria is strictly anaerobic and most of them are uncultivable in the laboratory (Dowd *et al.* 2008, Lamendella *et al.* 2011, Sommer *et al.* 2013). Hence, it is difficult to reproduce both the environmental conditions and the symbiotic relationships among micro-organisms found in the gut (Nocker *et al.* 2007). In contrast, the total intestinal microbiota is potentially affected by the presence of any antimicrobial. This makes that the study of antimicrobial selection in the microbiota remains a challenging topic. For this particular reason, we chose to start our research with an *in vitro* study (Chapter 4) using pure cultures of *E. coli,* a well-known bacterial species that is frequently used as an indicator. This study was then followed by more complicated *ex vivo* experiments with a model simulating the pig microbiota (Chapter 5), using an *E. coli* donor strain originating from the *in vitro* study. This had the advantage that the results obtained in more controlled conditions (Chapter 4) could be compared with the results obtained in less controlled but more realistic conditions (Chapter 5). Both studies proved the selective effect of 1 and 4 mg/l doxycycline (representing intestinal concentrations due to 1 and 3% cross-contamination) on resistant *tet*(A) carrying *E. coli* donor

strains. It is remarkable that the experimental set-up of both studies resulted in no significant difference between the selective effect of 1 and 4 mg/l doxycycline. In this context, it is important to underline that our resistant *E. coli* strains all carried *tet*(A), a tetracycline resistance gene that encodes the efflux pump TetA. This is an energy dependent resistance mechanism, which may influence the fitness of the bacteria carrying this mechanism. In other words, the fitness of these bacteria might decrease with increasing antimicrobial concentrations, which might explain why no difference in selective effect was found between 1 and 4 mg/l doxycycline. Other tetracycline resistance mechanisms (e.g. ribosomal protection, enzymatic inactivation) are not energy dependent, which may result in different selective effects. With this in mind, we conducted additional research, involving the faecal samples that were collected during the *in vivo* experiment with pigs (Chapter 3). Real-time PCR was performed on faecal samples of the blank control group and the doxycycline group to determine the abundancy of common tetracycline resistance genes in the porcine microbiota: *tet*(A), *tet*(B), *tet*(L) (encoding efflux pumps), *tet*(Q), *tet*(W), *tet*(O) and *tet*(M) (encoding ribosomal protection). In contrast with the studies in Chapter 4 and 5, no influence of doxycycline on *tet*(A) abundancy (present in approximately 1 out of 10^5 to 1 out of 10^4 of the total bacteria) was observed. This suggests that the complexity of the intestinal microbiota might 'mask' the impact of antimicrobials on resistance selection in certain bacterial groups of the intestinal microbiota. Only in case of the efflux pump encoding gene *tet*(L), a difference was observed between the control group and the doxycycline group. The abundancy of this gene rose by approximately 2 log units (from 10^{-4} to 10^{-2}) compared to the control group during the experimental period. It is currently difficult to find out the underlying reason for these observations, as these data could not yet be linked to for example abundancy of bacterial species. Several factors

may be involved. A first factor is the difference in bacterial species in which these genes are found: *tet*(A) is mostly associated with Gram negative bacteria such as Enterobacteriaceae, whereas *tet*(L) is found in Gram negative as well as Gram positive bacteria typical for the intestinal microbiota such as *Clostridium* spp., *Lactobacillus* spp., *etc.* A second factor is the genetic environment of the different genes and their ability to transfer horizontally. The *tet*(L) gene is generally found on small mobile plasmids, and occasionally becomes integrated in the chromosome (Roberts 1996). The *tet*(A) gene of the three *E. coli* strains used in our *in vitro* experiments was in all cases located on a transposon, which was associated with a mobile plasmid. This is the most common genetic location that has been reported for *tet*(A) and Gram negative tetracycline efflux genes in general.

Only one of the three plasmids (pEC292) seemed to be transferred more at 1 and 4 mg/l doxycycline compared to blank controls in the *in vitro* experiments. This was not observed *in vitro* for the resistant strain EC 682 (plasmid pEC682), which was also used as donor strain in the *ex vivo* experiments. In contrast, the results of the *ex vivo* experiments did suggest a broader dissemination of this plasmid both at 1 and 4 mg/l doxycycline supplementation. This highlights that plasmid transfer frequencies differ depending on the environment (Aminov 2011), and that conjugation dynamics are difficult to investigate due to confounding factors (Lopatkin *et al.* 2016). It has been suggested that the conditions in the gut microbiota such as the diversity and density of the microbial population may stimulate horizontal gene transfer (Aminov 2011, Salyers 1993). Furthermore, the selective effect on the resistant strain (EC682) and the resulting larger population of this strain, could have resulted in more opportunities for conjugation events and thus in a broader dissemination of the plasmid.

A recent study from our research group, using the same model for the pig caecum as in our *ex vivo* experiments, showed that the counts of resistant Enterobacteriaceae and cultivable anaerobes increased significantly upon administration of 4 or 16 mg/l doxycycline. Furthermore, no clear selective effect of 1 mg/l doxycycline on any of the resistant populations was found in this study (De Mulder *et al.* 2016), as was also the case for the resistant coliforms in Chapter 5. The diversity of bacteria and resistance mechanisms present in the total microbiota might have caused the different observations in counts of resistant coliforms or Enterobacteriaceae compared to the counts of the resistant *E. coli* strain (EC 682) in our pig caecum model.

In conclusion, our results showed that intestinal doxycycline concentrations due to cross-contaminated feed can select for *tet*(A) carrying *E. coli*, both *in vitro* as well as in a simulation model of the pig caecum.

2 RECOMMENDATIONS

2.1 REDUCE FEED MEDICATION

Carry-over of antimicrobials is a challenging issue to deal with. It is generally assumed that cross-contamination of feed is unavoidable, due to the technical and economic challenges the feed and pig industry face today. It seems likely that the risk of cross-contamination during the production, transport, storage and distribution of medicated feed, cannot be avoided totally, though it should be minimalized as much as possible. Hence, the best way to reduce the amount of cross-contaminated feed would be a **reduction in the production of medicated feed**.

A first important step to achieve a reduction would be the **prohibition of prophylactic antimicrobial treatments** (and growth promoters outside the EU). It has already been shown that prophylactic treatments can be avoided through improved herd management, vaccination schemes and biosecurity. Postma and co-authors showed that improving herd management and biosecurity (e.g. appropriate anthelminthic therapy, vaccination strategies, hygiene standards etc.) resulted in a reduction of antimicrobial usage of 52% in finisher pigs (from birth to slaughter) and 32% in breeding animals and even improved production results. Alternatively, the posology of existing antimicrobials could also be revised and optimized, since the current posology is mainly established solely on clinical efficacy, without taking resistance selection of pathogenic or commensal bacteria into account. This could lower the recommended dose and thus the possible carry over quantities. The Belgian FPS-funded project DOSERESIST "Influence of dosage, administration route and intestinal health on antibacterial resistance selection in pig intestinal commensal flora and optimization of

dosage regimen of selected antibacterial drugs" might amongst others contribute to this topic.

In case an antimicrobial group treatment is unavoidable, the **choice of the antimicrobial** should always be based on a correct laboratory identification of the bacterial pathogen followed by an accurate assessment of its *in vitro* antimicrobial susceptibility. Unfortunately, in practice, this kind of laboratory testing is often too expensive or time consuming, which forces the veterinarian to choose an antimicrobial based on the clinical diagnosis and his/her experience. This experience can be increased and supported by regular susceptibility testing, even when the result comes late and a treatment had to be started.

The **route of administration** should be by preference parenteral injection when a limited number of animals needs to be treated. In this respect, future research could be focussed on the early detection of infectious diseases using alternative and innovative biomarkers. Depending on the epidemiology of the pathogen, in particular the ease of spread, the early detected infected animals could be separated and treated individually.

When group medication is unavoidable, **alternatives for feed medication** that involve less risk on cross contamination should be pursued, provided that other possible risks of the alternative administration are investigated thoroughly.

Drinking water or milk medication may be an alternative for group medication. The most important arguments in favour of water medication are the flexibility of the therapy and the opportunity to select smaller groups of animals for therapy. Another advantage is that water uptake of sick animals tends to be better than feed uptake. Unfortunately, a switch to water medication often implies high costs for the farmer, due to the high quality drinking water system that would need to be introduced in most

farms. Furthermore, more data on the risk of environmental contamination, resistance development in the drinking water systems, drinking behaviour of the animals during medication and the risk of improper use of the medication by the farmer are needed to be able to finally conclude if water medication is the best method for group administration (Schouten 2011, van Krimpen 2007). Also, the commercial drug formulations used need 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.

In conclusion, water medication could be an acceptable alternative provided that technical superior and well maintained drinking water installations are used, drinking water quality and pharmaceutical drug formulations used are optimal, and spilling of medicated water into the environment is avoided as much as possible. In some countries, e.g. The Netherlands, Germany and Denmark, water medication is already preferred in pig industry and feed medication is not or rarely applied.

2.2 REDUCE THE RISK OF CROSS-CONTAMINATION

Besides a reduction of feed medication, the lowest possible risk of carry-over during production, transport and storage practices of medicated feed should be pursued. Apart from the GMP guidelines that always need to be applied during the production of medicated feed, some extra measurements can be taken to reduce the risk of carry-over. At the **feed mill**, carry-over can be reduced by targeting the practices (flushing after medicated feed production), the process (reducing the process length), the feed (sequencing medicated feed production and/or the active product used, choosing less- or non-dust producing products. The risk of cross-contam-

ination can be avoided considerably by mixing at the end of the feed line instead of in the main mixing line, as is in the case in Belgium (Filippitzi *et al.* 2016). Carry-over during **transport and delivery** can be minimised by the use of new, modified trucks, the use of back bins that reduce the length of the circuit and careful purging after each delivery.

A more recent practice of medicated feed production is the **fine dosing system (FDS) truck**. In this case, cross-contamination at the feed mill is avoided because the drug is mixed with the feed at the time of delivery at the farm. Compared to top dressing/mixing, the FDS truck has the advantage of a better homogeneity of the feed. The use of these trucks is however still limited because of their high cost. Moreover, also appropriate calibration of the FDS system is mandatory.

Cross-contamination at the **farm** mainly depends on the farmers' practices. Consequently, raising their and their personnel's awareness on the importance of purging is essential to minimise carry-over at the farm. Besides that, a separate feed silo for medicated feed would be optimal (Filippitzi *et al.* 2016). Feed medication via **top dressing/mixing** of the antimicrobial at the farm rules out cross-contamination at the feed mill or during transport and storage, but most likely results in improper dosage due to inhomogeneity of the medicated feed and is therefore not recommended (FASFC 2013, Schouten 2011).

2.3 ESTABLISH NEW REGULATION

Keeping in mind the One Health concept, the ban of the use of subtherapeutic antimicrobials as growth promoters, which was established in Europe in 2006, should be extended to a worldwide ban. A second step that would lead to a great reduction of antimicrobial use is the prohibition of prophylactic treatments. Unfortunately, these kind of global measure-

ments are likely to be held back or postponed because of economic interests. For example, it is suggested that the rising incomes in countries such as Brazil, Russia, India, and South-Africa will cause a shift from extensive farming systems to large-scale intensive farming operations that use antimicrobials in subtherapeutic doses for growth promoting purposes (Van Boeckel *et al.* 2015).

The establishment of legal limits for carry-over of antimicrobials in feed should have a beneficial effect, as this would reduce the quantity of antimicrobial residues in the environment in general. In 2014, the European Commission adopted a proposal for a regulation on the manufacture, placing on the market and use of medicated feed and repealing Council Directive, because the rules dating from 1990 (90/167/EEC) needed to be updated. One of the changes proposed, is the introduction of a general limit for carry-over of antimicrobials of 1% of the active substance of the last produced batch of medicated feed. It needs to be mentioned though that several agencies such as AGRI (Committee on Agriculture and Rural Development), FEFAC (European Feed Manufacturers' Federation) and COPA-COGECA (European farmers and cooperatives foundation) question the proposed 1% limit because they claim it is not feasible in practice and because they find that the proposed limits should be based on a scientific risk assessment. The European Consumer Organisation (BEUC) even suggests a total ban of antimicrobials in feed (EC 2016). When introducing the 1% limit, some practical concerns arise. The control of the carry-over levels at a feed mill is complicated by the fact that flushing batches are very inhomogeneous (Stolker *et al.* 2013). Consequently, very thorough sampling and controls of flushing batches would be needed to be sure if the proposed maximum limit of 1% is achieved. Because of these observations, NEVEDI (Association of Dutch Feed Producers), as the first in Europe, voluntarily decided to stop the production of medicated feed in feed mills

in 2011. They concluded that the production of medicated feed compliant with new strict standards regarding carry-over would be too expensive to keep it profitable.

Regulatory authorities should focus in the first place on guidelines and regulations to reduce the use of medicated feed, and second on feasible standards to reduce cross-contamination of feed. For example, the prohibition of prophylactic feed medication would be a straightforward approach that can reduce feed medication substantially and consequently reduce the risk on resistance selection. Avoiding cross-contamination of feed is equally important, as this also reduces the spread of antimicrobial residues into the environment. However, several questions arise when it comes to establishing legal limits for cross-contamination of feed. What is a feasible maximum level of carry-over that can be achieved in practice? As mentioned before, some feed producers doubt that strict rules regarding carry-over are feasible from an economical point of view. And more important, which maximum carry-over level will have a clear beneficial effect when it comes to resistance selection? In this thesis, it was concluded that a 1% carry-over level of doxycycline possibly does not reduce the risk on resistance selection substantially compared to a 3% carry-over level. Furthermore, other studies indicate that even antimicrobial concentrations a 100-fold lower can still select for resistant bacteria (Gullberg *et al.* 2011). Presumably, the selective pressure will also depend on the type of antimicrobial and the bacteria they are exposed to.

Another approach could be to prohibit the use of antimicrobials that cause the highest risk on resistance selection as feed medication. More research would be needed to identify these antimicrobials.

3 FUTURE RESEARCH

It was shown in this doctoral thesis that carry-over of antimicrobials in pig feed has an influence on resistance selection in the microbiota. Hence, carry-over should be avoided at all times. However, many questions remain and the following future research subjects can help to further elucidate the impact of cross-contamination on the spread of antimicrobial resistance.

— First of all, there is a lack of data on intestinal antimicrobial concentrations after oral administration of cross-contamination levels of antimicrobials. Attention should go first to this basic information, in particular concerning antimicrobials that are commonly applied as feed medication. The tested antimicrobials in this thesis are a useful start, but other typical feed medication antimicrobials (e.g. amoxicillin) should be investigated in the near future. A cross-contamination level of the feed of 1% may be a good choice for the experimental set-up, as this level is considered by the EU to be established as maximum carry-over level. However, a range of concentrations should be tested to be able to make extrapolations to different contamination levels.
— Regarding residual antimicrobial concentrations in the gut in general, also parenteral dosing should be tested, since it was demonstrated that certain antimicrobials such as sulphadiazine are secreted in the gut, with possible ion-trapping in the intestinal content.
— More information is needed with respect to antimicrobial concentration levels in the feed and drinking water when mixed at the farm. To what extent cross-contamination occurs at the pig farm after administration of antimicrobials in feed and drinking water? What are the

critical factors of the entire chain, from production to consumption of feed and drinking water with added drugs, to guarantee optimal drug delivery in pigs and to control cross-contamination?

— In this doctoral thesis, we focussed on two specific antimicrobial concentrations, caused by a 1 and 3% cross-contamination level, to evaluate the effect on resistance selection. Another interesting approach would be to determine MSC of selected antimicrobials for a selection of indicator bacteria (e.g. *E. coli, Enterococcus spp*.) that harbour different resistance mechanisms as they may have a different MSC and thus the selection for resistance may be different. It is thus crucial to include the different known resistance mechanisms. We investigated selection of *tet*(A) carrying bacteria, an energy dependent efflux mechanism, but we have no information on the selection of other (non-energy dependent) tetracycline resistance mechanisms such as ribosomal protection and enzymatic inactivation. All tetracycline inactivators cause high MIC values for tetracycline and oxytetracycline compared to the other tetracycline resistance mechanisms, and may thus be less prone to be selected at very low antimicrobial concentrations (Forsberg *et al.* 2015). Comparing the resulting MSCs with the intestinal concentrations that are found at a 1% cross contamination level would give a first indication of the effect of the establishment of a 1% carry-over limit for antimicrobials.

— After the *in vitro* determination of the MSCs for different indicator bacteria, the effect of these concentrations on the intestinal microbiota should be investigated using the porcine caecum model that was described in this thesis. There is however a need for more detail in these studies. The newly developed Exon-Primed Intron-Crossing (EPIC)-PCR allows to link functional genes and phylogenetic markers in uncultured single cells (Spencer *et al.* 2016), and thus makes it

possible to make a link between increase or decrease of a resistance gene with an increase or decrease in abundancy of bacterial species. In this way, the impact of the antimicrobial selection pressure can be assessed more in detail: which resistance genes are prone to selection and/or transfer, which bacterial species carry these genes, are the genes transferred to potential pathogenic bacterial species, etc. At the same time, taxonomical shifts in the microbiota can be evaluated in order to estimate the impact on the intestinal health of the animals. In these models, it will also be possible to study the persistence of resistance after termination of the drug exposure.

4 CONCLUSION

This doctoral thesis indicates that lowering legal maximum levels of carry-over of antimicrobials may not be the most efficient or fastest way to counter resistance selection in pigs. Our results show that 1% carry-over of doxycycline in the feed does not select significantly less for resistance compared to a 3% carry-over level. It is not yet clear if this also accounts for other antimicrobials. In our opinion, authorities should firstly use their resources to promote preventive health measures in order to reduce the total use of antimicrobials in pig production. Meanwhile, the (complex) research on the effect of cross-contaminated feed can be continued.

SUMMARY

Cross-contamination of feed with low concentrations of antimicrobials is an inevitable problem that results from the application of feed medication in the pig industry. As a result, a large number of pigs can be exposed to low concentrations of antimicrobials via the feed. To date, little is known about the possible effects of these low antimicrobial concentrations on the intestinal microbiota concerning resistance selection.

The **General Aim** of this doctoral thesis (Chapter 2) was to investigate resistance selection at the level of the intestinal microbiota when pigs consume feed that contains carry-over levels of antimicrobials.

Therefore, the intestinal concentrations after feeding with cross-contaminated feed needed to be determined first. In **Chapter 3,** an animal experiment was set up to determine the intestinal concentrations of chlortetracycline, doxycycline and sulphadiazine-trimethoprim in pigs when they are fed with experimental feed that contains 3% carry-over concentrations of these antimicrobials. Three groups of six pigs were fed during 10 days with an experimental feed that contained a 3% carry-over concentration of the three antimicrobials respectively. Another six pigs were fed blank feed and served as control group. The carry-over level of 3% was based on the only legal limit for carry-over valid at the time, as was set for coccidiostats (2009/8/EG). During the 10-day experimental period, faeces samples were collected every two days and at the end the animals were euthanized and samples of caecal and colonic content were taken. In general, relatively high concentrations were detected for tetracyclines and relatively low concentrations were found for sulphadiazine. A mean concentration of 10 mg/kg chlortetracycline and 4 mg/kg doxycycline in the faeces was reached, whereas mean sulphadiazine concentration was 0.7 mg/kg. Trimethoprim values were below the limit of detection of the analytical method. The mean transfer ratio's from feed to intestinal content (= ratio

of intestinal content concentration to feed concentration) were 39% for doxycycline, 82% for chlortetracycline and 4.1% for sulphadiazine. Transfer ratios were not calculated for trimethoprim. The transfer ratio's correspond well with the oral bioavailability of these drugs in pigs. In short, this study showed that the low to moderate oral bioavailability of tetracyclines can result in relatively high concentrations of these drugs in the caecum, colon and faeces of pigs, even at a 3% carry-over level in the feed.

The next step was to determine the effect of the intestinal concentrations found for doxycycline on resistance selection, first by means of an *in vitro* model with pure bacterial cultures of *Escherichia coli* (**Chapter 4**) and second with an *ex vivo* model of the porcine caecum (**Chapter 5**). Doxycycline was chosen based on the results of the *in vivo* experiment (high intestinal concentrations of tetracyclines) and because it is frequently applied as feed medication. Considering the recently established regulation on maximum carry-over limits in Belgium (1%), intestinal concentrations corresponding to 1% as well as 3% were included in these bacteriological studies. In both studies, the selective effect of 1 mg/l (corresponding with a carry-over concentration of approximately 1%) and of 4 mg/l doxycycline (corresponding with a carry-over concentration of approximately 3%) was compared with the selective effect of a blank medium.

In **Chapter 4** an *in vitro* experiment was conducted to determine whether and to what extent a doxycycline resistant *E. coli* is selected when it has to compete with a fully susceptible *E. coli* strain, in three different media (1 mg/l doxycycline, 4 mg/l doxycycline and blank medium). Three different resistant *E. coli* and four different susceptible *E. coli* strains were used. Each experiment was done in triplicate and involved one resistant strain that was brought in competition with one susceptible strain (initial ratio resistant/susceptible: 1/1000). Numbers of resistant and susceptible bacteria were counted after 24h and 48h of competition and the selective

effect of the medium was then calculated as the ratio resistant bacteria/susceptible bacteria. The results showed that the medium with 1 mg/l doxycycline as well as the medium with 4 mg/l doxycycline had a significant higher selective effect than the blank medium. Moreover, no significant difference in selective effect between 1 mg/l doxycycline and 4 mg/l doxycycline was observed. In addition, transconjugants (initially susceptible bacteria that have acquired resistance from the resistant strain) were counted in part of the experiments to determine the transfer rate of the resistance plasmids. Only one of the three plasmids (pEC292) showed a higher transfer rate at 1 and/or 4 mg/l doxycycline, dependent on the recipient strain.

In **Chapter 5**, the caecal microbiota of the pig was simulated using a bioreactor. The bioreactors were inoculated with pooled caecal content of organic raised pigs and a pure culture of the known resistant *E. coli* strain (EC682) from the *in vitro* study (Chapter 4). After 24h of batch fermentation, the continuous fermentation was started. Forty-eight hours later, doxycycline was administered via the medium (1 mg/l or 4 mg/l). For each condition, the experiment was done in triplicate. Growth of the *E. coli* donor strain (EC 682) was monitored during ten days at three different conditions: 1 mg/l doxycycline, 4 mg/l doxycycline or blank medium. Furthermore, 420 doxycycline resistant *E. coli*, isolated from the different experiments, were characterized to verify whether they acquired the plasmid of donor strain EC 682. Both 1 mg/l and 4 mg/l doxycycline showed to have a significant higher selective effect on EC 682 compared to blank medium. Remarkably, no significant difference in selective effect was found between 1 mg/l and 4 mg/l doxycycline, similarly as in the *in vitro* study (Chapter 4). Characterization of the 420 *E. coli* isolates from the different reactor runs showed that plasmid pEC682 was transferred to other *E. coli* strains in all three conditions (0, 1 and 4 mg/l doxycycline). In absolute numbers, more transconjugants (*E. coli* that received pEC682) were isolated from the

experiments with 1 mg/l and 4 mg/l doxycycline compared to the blank control experiments. It is plausible that the larger numbers of transconjugants are caused by a higher transfer frequency due to the selective pressure of doxycycline or the enrichment of transconjugants due to this selective pressure. Additional experiments would however be needed to confirm the observations on transfer frequency statistically.

In conclusion, this doctoral thesis showed that the poor oral bioavailability of tetracyclines results in relatively high concentrations in caecal and colonic content and faeces at steady-state conditions, even at a carry-over level of 3% in the feed. *In vitro* experiments with *E. coli* as well as *ex vivo* experiments simulating the pig caecum showed that intestinal doxycycline concentrations corresponding with approximately 1% and 3% carry-over levels in the feed, both select for a *tet*(A) carrying doxycycline resistant *E. coli* strain compared to a blank control.

SAMENVATTING

Kruiscontaminatie van voeders met lage concentraties antimicrobiële middelen is een onvermijdelijk probleem dat voortvloeit uit de toepassing van voedermedicatie in de varkenssector. Als gevolg van dit fenomeen komt een aanzienlijk aantal varkens in contact met lage dosissen antimicrobiële middelen via het voeder. Er bestaat nog veel onduidelijkheid over de mogelijke effecten van deze lage concentraties op resistentieselectie in de intestinale microbiota.

De **Algemene Doelstelling** van dit doctoraatsonderzoek (Hoofdstuk 2) was de resistentieselectie te onderzoeken ter hoogte van de intestinale microbiota van het varken na opname van voeder dat verslepingsconcentraties van antimicrobiële middelen bevat.

In **Hoofdstuk 3** werd tijdens een dierproef bepaald wat de concentratie van antimicrobiële middelen is in de darm van varkens wanneer er gevoederd wordt met lage dosissen van deze geneesmiddelen. Zowel tetracyclines (chloortetracycline en doxycycline) als sulphadiazine-trimethoprim werden onderzocht. Drie groepen van telkens zes varkens werden gedurende 10 dagen gevoederd met experimenteel voeder dat een verslepingsconcentratie van 3% bevatte van respectievelijk chloortetracycline, doxycycline en sulphadiazine-trimethoprim. Een derde groep van zes varkens diende als blanco controlegroep. De keuze voor het verslepingsniveau was gebaseerd op de wettelijke aanvaardbare grens van versleping van coccidiostatica (2009/8/EG). Gedurende de proefperiode werden om de 2 dagen individuele meststalen genomen en na 10 dagen werden de dieren geeuthanaseerd waarbij ook stalen van caecum- en coloninhoud werden genomen. Over het algemeen werden voor de tetracyclines relatief hoge concentraties teruggevonden, en voor sulphadiazine relatief lage concentraties. De gemiddelde concentratie in de mest was 10 mg/kg en 4 mg/kg

voor chloortetracycline en doxycycline, respectievelijk, terwijl voor sulphadiazine een concentratie van 0.7 mg/kg werd gemeten. De gehaltes voor trimethoprim lagen onder de detectielimiet van de analysemethode. De gemiddelde transfer ratio's van voeder naar darminhoud (= verhouding van concentratie in darminhoud tot de concentratie in het voeder) waren 39% voor doxycycline en 82% voor chloortetracycline. In het geval van sulphadiazine was dit 4,1%; de transfer ratio van trimethoprim werd niet berekend. Deze transfer ratio's komen goed overeen met de gekende orale biologische beschikbaarheden van deze geneesmiddelen bij varkens. Uit de resultaten kon besloten worden dat de lage biologische beschikbaarheden van tetracyclines kunnen leiden tot vrij hoge concentraties in caecum, colon en mest, zelfs bij een 3% kruiscontaminatie van het voeder.

Vervolgens werd aan de hand van bacteriologische experimenten, eerst *in vitro* met zuivere bacteriële culturen van *Escherichia coli* (**Hoofdstuk 4**) en vervolgens met een *ex vivo* model dat de microbiota van het varkenscaecum nabootst (**Hoofdstuk 5**), de invloed van lage concentraties aan doxycycline op resistentieselectie en -transfer getest. Doxycycline werd gekozen gebaseerd op de resultaten van de *in vivo* proef (hoge intestinale concentraties van tetracyclines) en omdat dit geneesmiddel vaak toegediend wordt onder de vorm van voedermedicatie. Wegens de recente ontwikkelingen in de regelgeving rond versleping, werd er bij deze proeven naast het verslepingsniveau van 3% ook rekening gehouden met een verslepingsniveau van 1%. Er werd nagegaan hoe groot het selecterend effect is van 1 mg/l (corresponderend met een versleping van ongeveer 1%) en 4 mg/l doxycycline (corresponderend met een versleping van ongeveer 3%), in vergelijking met een blanco milieu.

In **Hoofdstuk 4** werd een *in vitro* experiment opgezet waarbij werd nagegaan of en in welke mate een resistente *E. coli* stam uitgeselecteerd wordt wanneer deze in competitie gebracht wordt met een niet-resistente

E. coli stam, bij de twee doxycycline concentraties en ter controle in een blanco milieu. Deze testen werden uitgevoerd met drie verschillende resistente *E. coli* stammen en vier gevoelige *E. coli* stammen, waarbij telkens één resistente met één gevoelige stam in competitie gebracht werd (initiële verhouding resistent/gevoelig: 1/1000). Resistente en gevoelige kiemen werden geteld na 24u en na 48u competitie en het selectief effect van het medium werd vervolgens berekend als de verhouding van het aantal resistente/aantal gevoelige kiemen. Hieruit bleek dat zowel het milieu met 1 mg/l als met 4 mg/l doxycycline een significant hoger selectief effect heeft dan een blanco milieu. In blanco milieu groeit de gevoelige stam verder uit ten koste van de resistente stam. In de doxycycline milieus echter, keert de initiële verhouding van 1/1000 (resistent/gevoelig) om, en krijgt de resistente stam de overhand. Slechts voor één resistente stam werd een significant hogere transfer frequentie van het resistentieplasmide (= aantal transconjuganten/totaal aantal recipiënten) bij 1 mg/l of een significant hogere transfer bij 1 mg/l en 4 mg/l doxycycline waargenomen, afhankelijk van de recipiënt stam. Bij de andere resistente stammen werden geen significante verschillen waargenomen tussen de transfer frequenties in de verschillende milieus.

In **Hoofdstuk 5** werden bioreactors gebruikt om het caecum van het varken te simuleren. Deze werden geïnoculeerd met gepoolde caecale inhoud van biologische varkens en op hetzelfde moment met een pure cultuur van een gekende doxycycline resistente *E. coli* uit Hoofdstuk 4 (EC12 682). Na een 24h batch cultuur werd het continue fermentatiesysteem opgestart. Vervolgens werd 48h later deze microbiota verstoord door het toedienen van doxycycline via het medium (1 mg/l of 4 mg/l). De groei van de gekende *E. coli* werd opgevolgd gedurende 10 dagen onder drie verschillende condities: 1 mg/l doxycycline, 4 mg/l doxycycline en een blanco controle. Per conditie werd het experiment 3 maal herhaald om een trend te

kunnen waarnemen. Daarnaast werden 420 doxycycline resistente *E. coli*'s, die geïsoleerd werden uit deze experimenten, moleculair gekarakteriseerd om na te gaan of zij hun resistentie al dan niet via de donorstam verworven hadden. Zowel supplementatie van het medium met 1 als met 4 mg/l doxycycline had een selectief effect op de donor, maar er werd geen significant verschillend selectief effect tussen 1 en 4 mg/l doxycycline aangetoond. De karakterisatie van 420 doxycycline resistente *E. coli's* uit de verschillende reactor runs toonde aan dat plasmide pEC682 getransfereerd werd van de donor naar andere *E. coli* stammen onder elke conditie (0, 1 en 4 µg/ml doxycycline). Er werden echter duidelijk meer transconjuganten geïsoleerd uit zowel de experimenten met 1 mg/l doxycycline als deze met 4 mg/l doxycycline vergeleken met de blanco experimenten. Dit groter aantal kan te wijten zijn aan ofwel een hogere transfer frequentie van plasmide onder de selectieve druk van doxycycline, ofwel het aanrijken van transconjuganten onder deze selectieve druk. Meer uitgebreide experimenten zijn echter nodig om de waarnemingen rond transfer statistisch te bevestigen.

Dit doctoraatsonderzoek toonde aan dat de lage orale biologische beschikbaarheid bij varkens van tetracyclines kan leiden tot relatief hoge concentraties in de darm, zelfs bij verslepingsconcentraties in het voeder van 3%. Zowel de *in vitro* experimenten met *E. coli* als *ex vivo* simulaties van het varkenscaecum toonden aan dat intestinale concentraties doxycycline ten gevolge van ongeveer 1% en 3% versleping in het voeder, beiden een *tet*(A) dragende, doxycycline resistente *E. coli* stam uitselecteren vergeleken met een blanco controle.

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REFERENCES

AACTING (2018). Network on quantification of veterinary Antimicrobial usage at herd level and Analysis, CommunicaTion and benchmarkING to improve responsible usage. Monitoring systems. http://www.aacting.org/monitoring-systems/.

Agerso, H. and Friis, C. (1998). Bioavailability of amoxycillin in pigs. *Journal of Veterinary Pharmacology and Therapeutics* **21**(1): 41-46.

Agga, G. E., Scott, H. M., Vinasco, J., Nagaraja, T. G., Amachawadi, R. G., Bai, J. F., Norby, B., Renter, D. G., Dritz, S. S., Nelssen, J. L. and Tokach, M. D. (2015). Effects of chlortetracycline and copper supplementation on the prevalence, distribution, and quantity of antimicrobial resistance genes in the fecal metagenome of weaned pigs. *Preventive Veterinary Medicine* **119**(3-4): 179-189.

Ahrens, F. A. and Martin, R. J. (2008) Antimicrobial drugs. In *Handbook of Veterinary Pharmacology*
ed. Hsu, W. H. p.357. Ames, Iowa, U.S.: Wiley-Blackwell.

Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L. and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* **12**(1): 1-10.

Allmeier, H., Cresnar, B., Greck, M. and Schmitt, R. (1992). Complete nucleotide sequence of Tn1721: gene organization and a novel gene product with features of a chemotaxis protein. *Gene* **111**(1): 11-20.

AMCRA (2013). Center of Expertise on Antimicrobial Consumption and Resistance in Animals. Gids voor goed gebruik van antibacteriële middelen in de varkenshouderij. http://amcra.be/en/gidsen-goed-gebruik.

AMCRA (2014). Center of Expertise on Antimicrobial Consumption and Resistance in Animals. Visie 2020. http://www.amcra.be/nl/nieuws/amcra-visie-2020.

Aminov, R. I. (2011). Horizontal Gene Exchange in Environmental Microbiota. *Frontiers in Microbiology* **2**: 158.

Andersson, D. I. and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology* **8**(4): 260-271.

Andersson, D. I. and Hughes, D. (2011). Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiology Reviews* **35**(5): 901-911.

Andersson, D. I. and Hughes, D. (2012). Evolution of antibiotic resistance at non-lethal drug concentrations. *Drug Resistance Updates* **15**(3): 162-172.

Andersson, D. I. and Hughes, D. (2014). Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology* **12**(7): 465-478.

Andersson, D. I. and Levin, B. R. (1999). The biological cost of antibiotic resistance. *Current Opinion in Microbiology* **2**(5): 489-493.

Aune, T. E. and Aachmann, F. L. (2010). Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. *Applied Microbiology and Biotechnology* **85**(5): 1301-1313.

Australian Government (2014). Report on the Quantity of Antimicrobial Products Sold for Veterinary Use in Australia: July 2005 to June 2010. http://apvma.gov.au/node/11816.

Baert, K., Croubels, S., Gasthuys, F., De Busser, J. and De Backer, P. (2000). Pharmacokinetics and oral bioavailability of a doxycycline formulation (DOXYCYCLINE 75%) in nonfasted young pigs. *Journal of Veterinary Pharmacology and Therapeutics* **23**(1): 45-48.

Baert, K., De Baere, S., Croubels, S., Gasthuys, F. and De Backer, P. (2001). Pharmacokinetics and bioavailability of sulfadiazine and trimethoprim (trimazin 30%) after oral administration in non-fasted young pigs. *Journal of Veterinary Pharmacology and Therapeutics* **24**(4): 295-298.

Balcazar, J. L. (2014). Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment. *Plos Pathogens* **10**(7): e1004219.

Baquero, F. and Negri, M. C. (1997a). Strategies to minimize the development of antibiotic resistance. *Journal of Chemotherapy* **9 Suppl 3**: 29-37.

Baquero, F., Negri, M. C., Morosini, M. I. and Blazquez, J. (1997b). The antibiotic selective process: concentration-specific amplification of low-level resistant populations. *Ciba Found Symp* **207**: 93-105; discussion 105-111.

Barkovskii, A. L. and Bridges, C. (2012). Persistence and profiles of tetracycline resistance genes in swine farms and impact of operational practices on their occurrence in farms' vicinities *Water Air and Soil Pollution* **223**(6): 3577-3577.

Barr, V., Barr, K., Millar, M. R. and Lacey, R. W. (1986). Beta-lactam antibiotics increase the frequency of plasmid transfer in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **17**(4): 409-413.

Bates, D., Machler, M., Bolker, B. M. and Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67**(1): 1-48.

Beaber, J. W., Hochhut, B. and Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**(6969): 72-74.

Bell, B. G., Schellevis, F., Stobberingh, E., Goossens, H. and Pringle, M. (2014). A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. *BMC Infectious Diseases* **14**: 13.

BelVetSAC (2013). Belgian Veterinary Surveillance of Antimicrobial Consumption National consumption report 2012. http://www.belvetsac.ugent.be/pages/home/BelvetSAC_report_2012%20finaal.pdf.

BelvetSAC (2015). Belgian Veterinary Surveillance of Antibacterial Consumption National consumption report 2014. http://www.belvetsac.ugent.be/BelvetSAC_report_2014.pdf.

BelVetSAC (2016). Belgian Veterinary Surveillance of Antibacterial Consumption National consumption report 2015. www.belvetsac.ugent.be/pages/home/BelvetSAC_report_2015%20finaal.pdf.

BelVetSAC (2017). Belgian Veterinary Surveillance of Antibacterial Consumption National consumption report 2016 http://www.belvetsac.ugent.be/BelvetSAC_report_2016.pdf.

Bengtsson-Palme, J. and Larsson, D. G. J. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environment International* **86**: 140-149.

Berendsen, B. J., Wegh, R. S., Memelink, J., Zuidema, T. and Stolker, L. A. (2015). The analysis of animal faeces as a tool to monitor antibiotic usage. *Talanta* **132**: 258-268.

Bernabeu, J. A., Torres-Suarez, A. I., Gil-Alegre, M. E. and Camacho, M. A. (2000). Stability study of a sulfadimidine-containing medicated premix and its mixture with farm feed. *Journal of Agricultural and Food Chemistry* **48**(12): 5969-5974.

Bertrand, S., Weill, F. X., Cloeckaert, A., Vrints, M., Mairiaux, E., Praud, K., Dierick, K., Wildemauve, C., Godard, C., Butaye, P., Imberechts, H., Grimont, P. A. and

Collard, J. M. (2006). Clonal emergence of extended-spectrum beta-lactamase (CTX-M-2)-producing Salmonella enterica serovar Virchow isolates with reduced susceptibilities to ciprofloxacin among poultry and humans in Belgium and France (2000 to 2003). *Journal of Clinical Microbiology* **44**(8): 2897-2903.

BFA and FASFC (2013). Convenant betreffende gemedicineerde voeders. www.favv.be/extra/convenant/_documents/2013-01-30_CONVENANT-FAVV-BEMEFA-gemedicineerde-voeders_finaal_jan-2013_NL.pdf.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. and Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* **13**(1): 42-51.

Blanco, M., Gutiérrez-Martin, C. B., Rodríguez-Ferri, E. F., Roberts, M. C. and Navas, J. (2006). Distribution of Tetracycline Resistance Genes in Actinobacillus pleuropneumoniae Isolates from Spain. *Antimicrobial Agents and Chemotherapy* **50**(2): 702-708.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M. B. and Martinez, J. L. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. *Microorganisms* **4**(1).

Boerlin, P. and Reid-Smith, R. J. (2008). Antimicrobial resistance: its emergence and transmission. *Animal Health Research Reviews* **9**(2): 115-126.

Boothe, D. M. (2016). Sulfonamides and Sulfonamide Combinations. Merck Veterinary Manual http://www.merckvetmanual.com/pharmacology/antibacterial-agents/sulfonamides-and-sulfonamide-combinations.

Borras, S., Companyo, R., Granados, M., Guiteras, J., Perez-Vendrell, A. M., Brufau, J., Medina, M. and Bosch, J. (2011). Analysis of antimicrobial agents in animal feed. *Trac-Trends in Analytical Chemistry* **30**(7): 1042-1064.

Brewer, M. T., Xiong, N., Anderson, K. L. and Carlson, S. A. (2013). Effects of subtherapeutic concentrations of antimicrobials on gene acquisition events in *Yersinia*, *Proteus*, *Shigella*, and *Salmonella* recipient organisms in isolated ligated intestinal loops of swine. *American Journal of Veterinary Research* **74**(8): 1078-1083.

Brouwer, M. S., Bossers, A., Harders, F., van Essen-Zandbergen, A., Mevius, D. J. and Smith, H. E. (2014). Complete Genome Sequences of IncI1 Plasmids Carrying Extended-Spectrum beta-Lactamase Genes. *Genome Announcements* **2**(4).

Burch, D. (2011). Potential effects of carryover of antimicrobials in pig feed. http://www.octagon-services.co.uk/articles/carryover.pdf.

Burch, D. (2013) Antimicrobial drug use in swine. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.553-568. Hoboken, NJ: John Wiley & Sons, Inc.

Burch, D. G. S. (2005). Pharmacokinetic, pharmacodynamic and clinical correlations relating to the therapy of colonic infections in the pig and breakpoint determinations. . *the Pig Journal* **56**: 8-24.

Burch, D. G. S. and Klein, U. (2008). Pharmacokinetic/pharmacodynamic relationships of tiamulin (Denagard®) for proliferative enteropathu 'ileitis'. http://www.octagon-services.co.uk/articles/enteropathy.pdf.

Burrus, V., Pavlovic, G., Decaris, B. and Guedon, G. (2002). Conjugative transposons: the tip of the iceberg. *Molecular Microbiology* **46**: 601-610.

Bushby, S. R. (1980). Sulfonamide and trimethoprim combinations. *Journal of the American Veterinary Medical Association* **176**(10 Spec No): 1049-1053.

Butaye, P., Devriese, L. A. and Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on Gram positive bacteria. *Clinical Microbiology Reviews* **16**(2): 175-+.

Callens, B., Persoons, D., Maes, D., Laanen, M., Postma, M., Boyen, F., Haesebrouck, F., Butaye, P., Catry, B. and Dewulf, J. (2012). Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. *Preventive Veterinary Medicine* **106**(1): 53-62.

Callens, B., Sarrazin, S., Cargnel, M., Welby, S., Dewulf, J., Hoet, B., Vermeersch, K. and Wattiau, P. (2017). Associations between a decreased veterinary antimicrobial use and resistance in commensal Escherichia coli from Belgian livestock species (2011–2015). *Preventive Veterinary Medicine*, https://doi.org/10.1016/j.prevetmed.2017.10.013.

Cambray, G., Guerout, A. M. and Mazel, D. (2010). Integrons. *Annual Review of Genetics* **44**: 141-166.

Cameron, A. and McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. *Journal of Animal Science and Biotechnology* **7**: 68.

Carattoli, A. (2009). Resistance plasmid families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* **53**(6): 2227-2238.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L. and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods* **63**(3): 219-228.

Chantziaras, I., Boyen, F., Callens, B. and Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of Antimicrobial Chemotherapy* **69**(3): 827-834.

Charpentier, X., Kay, E., Schneider, D. and Shuman, H. A. (2011). Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*. *Journal of Bacteriology* **193**(5): 1114-1121.

Charpentier, X., Polard, P. and Claverys, J. P. (2012). Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS? *Current Opinion in Microbiology* **15**(5): 570-576.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N. and Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied and Environmental Microbiology* **67**(4): 1494-1502.

Chen, L., Song, Y., Wei, Z., He, H., Zhang, A. and Jin, M. (2013). Antimicrobial susceptibility, tetracycline and erythromycin resistance genes, and multilocus sequence typing of Streptococcus suis isolates from diseased pigs in China. *Journal of Veterinary Medical Science* **75**(5): 583-587.

Cherlet, M., De Backer, P. and Croubels, S. (2006). Control of the keto-enol tautomerism of chlortetracycline for its straightforward quantitation in pig tissues by liquid chromatography-electrospray ionization tandem mass spectrometry. *Journal of Chromatography A* **1133**(1-2): 135-141.

Cherlet, M., Schelkens, M., Croubels, S. and De Backer, P. (2003). Quantitative multi-residue analysis of tetracyclines and their 4-epimers in pig tissues by high-performance liquid chromatography combined with positive-ion electrospray ionization mass spectrometry. *Analytica Chimica Acta* **492**(1-2): 199-213.

Chopra, I. and Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* **65**(2): 232-260.

CIPARS (2016). Government of Canada. Canadian Integrated Program for Antimicrobial Resistance Surveillance 2014 Annual Report. Public Health Agency of Canada, Guelph, Ontario. http://www.phac-aspc.gc.ca/cipars-picra/2014/annu-report-rapport-eng.php.

Cogliani, C., Goossens, H. and Greko, C. (2011). Restricting Antimicrobial Use in Food Animals: Lessons from Europe. *Microbe Magazine*, doi:10.1128/microbe.6.274.1.

Cohen, S. P., McMurry, L. M. and Levy, S. B. (1988). marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. *Journal of Bacteriology* **170**(12): 5416-5422.

Collignon, P. C., Conly, J. M., Andremont, A., McEwen, S. A., Aidara-Kane, A., Grp, W. H. O. A. and Surveill, B. M. I. (2016). World Health Organization Ranking of Antimicrobials According to Their Importance in Human Medicine: A Critical Step for Developing Risk Management Strategies to Control Antimicrobial Resistance From Food Animal Production. *Clinical Infectious Diseases* **63**(8): 1087-1093.

Colomer-Lluch, M., Imamovic, L., Jofre, J. and Muniesa, M. (2011). Bacteriophages Carrying Antibiotic Resistance Genes in Fecal Waste from Cattle, Pigs, and Poultry. *Antimicrobial Agents and Chemotherapy* **55**(10): 4908-4911.

Connell, S. R., Tracz, D. M., Nierhaus, K. H. and Taylor, D. E. (2003). Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. *Antimicrobial Agents and Chemotherapy* **47**(12): 3675-3681.

Council of the European Communities (1990). Council Directive of 26 March 1990 laying down the conditions governing the preparation, placing on the market and use of medicated feeding-stuffs in the community (90/167/EEC). *Official Journal of the European Union* **92**: 42-48.

Counotte, G. H., Eefting, T. and Bosch, A. (1984). [Stability and distribution of oxytetracycline HCI during manufacture and storage of pig feed under field conditions]. *Tijdschr Diergeneeskd* **109**(9): 339-344.

Cunha, B. A., Domenico, P. and Cunha, C. B. (2000). Pharmacodynamics of doxycycline. *Clinical Microbiology and Infection* **6**(5): 270-273.

DANMAP (2017). Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. http://www.danmap.org.

Davison, J. (1999). Genetic Exchange between Bacteria in the Environment. *Plasmid* **42**(2): 73-91.

Dawson, K. A., Langlois, B. E., Stahly, T. S. and Cromwell, G. L. (1984). Antibiotic resistance in anaerobic and coliform bacteria from the intestinal tract of swine fed therapeutic and subtherapeutic concentrations of chlortetracycline. *Journal of Animal Science* **58**(1): 123-131.

De Mulder, T., Rasschaert, G., Van Coillie, E., Van de Wiele, T. and Heyndrickx, M. (2016). Impact of cross-contamination of feed with antimicrobial compounds on the microbial population in the gastrointestinal tract of pigs. BSFM. Brussels, Belgium.

De Smet, J., Croubels, S., De Backer, P. and Devreese, M. (2017). Effect of administration route and dose alteration on sulfadiazine-trimethoprim plasma and intestinal concentrations in pigs. *International Journal of Antimicrobial Agents*, 50(6):707-714.

del Castillo, J. R. E. (2013) Tetracyclines. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.257-268. Hoboken, NJ: John Wiley & Sons, Inc.

del Castillo, J. R. E., Elsener, J. and Martineau, G. P. (1998). Pharmacokinetic modeling of in-feed tetracyclines in pigs using a meta-analytic compartmental approach. *Swine Health and Production* **6**(5): 189-202.

Desmond Baggot, J. and Giguère, S. (2013) Principles of Antimicrobial Drug Bioavailability and Disposition. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.41-77. Hoboken, NJ: John Wiley & Sons, Inc.

Devreese, M., Antonissen, G., De Baere, S., De Backer, P. and Croubels, S. (2014). Effect of administration route and dose escalation on plasma and intestinal concentrations of enrofloxacin and ciprofloxacin in broiler chickens. *BMC Veterinary Research* **10**.

Domingues, S., da Silva, G. J. and Nielsen, K. M. (2012). Integrons: Vehicles and pathways for horizontal dissemination in bacteria. *Mobile Genetic Elements* **2**(5): 211-223.

Dorado-Garcia, A., Mevius, D. J., Jacobs, J. J., Van Geijlswijk, I. M., Mouton, J. W., Wagenaar, J. A. and Heederik, D. J. (2016). Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. *The Journal of Antimicrobial Chemotherapy* **71**(12): 3607-3619.

Dowd, S. E., Sun, Y., Wolcott, R. D., Domingo, A. and Carroll, J. A. (2008). Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. *Foodborne Pathogens and Disease* **5**(4): 459-472.

Doyle, D., McDowall, K. J., Butler, M. J. and Hunter, I. S. (1991). Characterization of an oxytetracycline-resistance gene, otrA, of *Streptomyces rimosus*. *Molecular Microbiology* **5**(12): 2923-2933.

Drlica, K. (2003). The mutant selection window and antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **52**(1): 11-17.

Drlica, K. and Zhao, X. L. (2007). Mutant selection window hypothesis updated. *Clinical Infectious Diseases* **44**(5): 681-688.

Dubnau, D. (1999). DNA uptake in bacteria. *Annu. Rev. Microbiol.* **53**: 217-244.

Duggar, B. M. (1948). Aureomycin; a product of the continuing search for new antibiotics. *Annals of the New York Academy of Sciences* **51**(Art. 2): 177-181.

EC (2001). Article 1(16) of Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. *Official Journal of the European Union* **311**: 7.

EC (2002). Commission Decision 2002/675/EC implementing Council Directive 96/23/EC concerning the performances of analytical methods and the interpretation of results. . *Official Journal of the European Union* **221**: 8-36.

EC (2011). European Commission. Communication from the Commission to the European Parliament and the Council - Action plan against the rising threats from Antimicrobial Resistance.

http://ec.europa.eu/dgs/health_consumer/docs/communication_amr_2011_748_en.pdf.

EC (2014). European Commission. Proposal for a regulation of the European Parliament and of the council on the manufacture, placing on the market and use of medicated feed and repealing Council Directive 90/167/EEC. http://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A52014PC0556.

EC (2015). European Commission. Commission Notice. Guidelines for the prudent use of antimicrobials in veterinary medicine. (2015/C 299/04). *Official Journal of the European Union* **C 299, 11.9.2015**: 7–26

EC (2016). European Union. Briefing EU legislation in progress. Review of medicated feed legislation. www.europarl.europa.eu/RegData/etudes/BRIE/2016/583843/EPRS_BRI%282016%29583843_EN.pdf.

EFSA (2009). Panel on Biological Hazards. Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic infections. EFSA Journal. http://dx.doi.org/10.2903/j.efsa.2009.1372.

EMA (2013). European Medicines Agency. Use of glycylcyclines in animals in the European Union: development of resistance and possible impact on human and animal health. https://www.google.be/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0ahUKEwjIuMnjnP7RAhUC9mMKHcAQBjYQFggiMAE&url=http%3A%2F%2Fwww.ema.europa.eu%2Fdocs%2Fen_GB%2Fdocument_library%2FReport%2F2013%2F07%2FWC500146814.pdf&usg=AFQjCNGli8R7rzAXf9qftoY5MSE6PNZYuw.

EMA (2015). European Surveillance of Veterinary Antimicrobial Consumption. Fifth ESVAC report. Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2013. (EMA/387934/2015), http://www.ema.europa.eu/docs/en_GB/document_library/Report/2015/10/WC500195687.pdf.

EMA (2016). European Surveillance of Veterinary Antimicrobial Consumption. Sixth ESVAC report. 'Sales of veterinary antimicrobial agents in 29 European countries in 2014' (EMA/61769/2016).

EMEA (1996). Note for guidance: Additional quality requirements for products intended for incorporation into animal feedingstuffs (medicated premixes).

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004466.pdf.

EP (2002). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Official Journal of the European Union* **140**: 10-21.

EP (2003). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union* **268**: 29-43.

EP (2005a). Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products. *Official Journal of the European Union* **311**: 1-66.

EP (2005b). Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. *Official Journal of the European Union* **35**: 1-22.

EP (2009). Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC. **229**: 1-28.

EPRUMA (2013). Document on veterinary medicinal product (VMP) terminology. http://www.epruma.eu/publications/factsheets/publication/32-epruma-document-on-veterinary-medicinal-product-terminology.html.

EPRUMA (2014). Administration of veterinary medicines. http://www.epruma.eu/benefits/administration-of-veterinary-medicines.html.

EUCAST. Antimicrobial susceptibility testing EUCAST disk diffusion method. Version 5.0, January 2015. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Manual_v_5.0_EUCAST_Disk_Test.pdf.

EURL-AR (2013). Cut-off values recommended by the EU Reference Laboratory for Antimicrobial Resistance. http://www.crl-ar.eu/data/images/faq/eurl-recommended%20cut%20off%20values-29-11-2013.pdf.

FAO (2011). Food and Agricultural Organisation of the United Nations. Guidelines for risk analysis of foodborne antimicrobial resistance. www.codexalimentarius.net/download/standards/11776/CXG_077e.pdf.

FAO and OIE (2008). Food and Agriculture Organization of the United Nations, World Organisation for Animal Health,World Health Organization, United Nation System Influenza Coordinator, United Nations Children's Fund,World Bank. Contributing to One World, One Health: A Strategic Framework for Reducing Risks of Infectious Diseases at the Animal-Human-Ecosystems Interface. Rome.

FASFC (2013). Federal Agency for the Safety of the Food Chain. Advice 19-2013 of the Scientific Committee of the FASFC on responsible use of antibacterial substances via group treatment of livestock and the effect on the selection of resistance. http://www.afsca.be/wetenschappelijkcomite/adviezen/_documents/ADVIES19-2013_NL_DossierSciCom2012-10.pdf.

FDA (2013). Guidance for Industry #213. New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #209. https://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM299624.pdf.

FDA (2016). US Food and Drug Administration. 2015 Summary report on antimicrobials sold or distributed for use in food producing animals. http://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates/ucm534244.htm.

Fernandez-Lopez, R., Garcillan-Barcia, M. P., Revilla, C., Lazaro, M., Vielva, L. and de la Cruz, F. (2006). Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. *FEMS Microbiology Reviews* **30**(6): 942-966.

Filippitzi, M. E., Sarrazin, S., Imberechts, H., Smet, A. and Dewulf, J. (2016). Risk of cross-contamination due to the use of antimicrobial medicated feed throughout the trail of feed from the feed mill to the farm. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **33**(4): 644-655.

Finlay, A. C., Hobby, G. L. and et al. (1950). Terramycin, a new antibiotic. *Science* **111**(2874): 85.

Finley, R. L., Collignon, P., Larsson, D. G. J., McEwen, S. A., Li, X. Z., Gaze, W. H., Reid-Smith, R., Timinouni, M., Graham, D. W. and Topp, E. (2013). The Scourge of

Antibiotic Resistance: The Important Role of the Environment. *Clinical Infectious Diseases* **57**(5): 704-710.

Forsberg, K. J., Patel, S., Wencewicz, T. A. and Dantas, G. (2015). The Tetracycline Destructases: A Novel Family of Tetracycline-Inactivating Enzymes. *Chemistry & Biology* **22**(7): 888-897.

Furuya, E. Y. and Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. *Nature Reviews Microbiology* **4**(1): 36-45.

Gerzova, L., Babak, V., Sedlar, K., Faldynova, M., Videnska, P., Cejkova, D., Jensen, A. N., Denis, M., Kerouanton, A., Ricci, A., Cibin, V., Osterberg, J. and Rychlik, I. (2015). Characterization of Antibiotic Resistance Gene Abundance and Microbiota Composition in Feces of Organic and Conventional Pigs from Four EU Countries. *Plos One* **10**(7): e0132892.

Giguère, S. (2013) Antimicrobial Drug Action and Interaction: An Introduction. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.3-10. Hoboken, NJ: John Wiley & Sons, Inc.

Gillings, M. R. (2014). Integrons: Past, Present, and Future. *Microbiology and Molecular Biology Reviews* **78**(2): 257-277.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M. and Zhu, Y. G. (2015). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME Journal* **9**(6): 1269-1279.

Gómez-Pacheco, C. V., Sánchez-Polo, M., Rivera-Utrilla, J. and López-Peñalver, J. J. (2012). Tetracycline degradation in aqueous phase by ultraviolet radiation. *Chemical Engineering Journal* **187**: 89-95.

Grabowski, T. and Jaroszewski, J. J. (2009). Bioavailability of veterinary drugs in vivo and in silico. *Journal of Veterinary Pharmacology and Therapeutics* **32**(3): 249-257.

Grohmann, E., Muth, G. and Espinosa, M. (2003). Conjugative plasmid transfer in Gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **67**: 277-301.

Grossman, T. H., Starosta, A. L., Fyfe, C., O'Brien, W., Rothstein, D. M., Mikolajka, A., Wilson, D. N. and Sutcliffe, J. A. (2012). Target- and resistance-based mechanistic studies with TP-434, a novel fluorocycline antibiotic. *Antimicrobial Agents and Chemotherapy* **56**(5): 2559-2564.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbe, J., Ploy, M. C. and Mazel, D. (2009). The SOS response controls integron recombination. *Science* **324**(5930): 1034.

Guillaume, G., Ledent, V., Moens, W. and Collard, J. M. (2004). Phylogeny of efflux-mediated tetracycline resistance genes and related proteins revisited. *Microbial Drug Resistance-Mechanisms Epidemiology and Disease* **10**(1): 11-26.

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L. and Andersson, D. I. (2014). Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *Mbio* **5**(5).

Gullberg, E., Cao, S., Berg, O. G., Ilback, C., Sandegren, L., Hughes, D. and Andersson, D. I. (2011). Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. *Plos Pathogens* **7**(7).

Gumpert, H., Kubicek-Sutherland, J. Z., Porse, A., Karami, N., Munck, C., Linkevicius, M., Adlerberth, I., Wold, A. E., Andersson, D. I. and Sommer, M. O. A. (2017). Transfer and Persistence of a Multi-Drug Resistance Plasmid in situ of the Infant Gut Microbiota in the Absence of Antibiotic Treatment. *Frontiers in Microbiology* **8**(1852).

Gustafson, R. H. and Bowen, R. E. (1997). Antibiotic use in animal agriculture. *Journal of Applied Microbiology* **83**(5): 531-541.

Hall, B. G., Acar, H., Nandipati, A. and Barlow, M. (2013). Growth Rates Made Easy. *Molecular Biology and Evolution* **31**(1): 232-238.

Hall, R. M., Brown, H. J., Brookes, D. E. and Stokes, H. W. (1994). Integrons found in different locations have identical 5' ends but variable 3' ends. *Journal of Bacteriology* **176**(20): 6286-6294.

Hamscher, G., Pawelzick, H. T., Sczesny, S., Nau, H. and Hartung, J. (2003). Antibiotics in dust originating from a pig-fattening farm: a new source of health hazard for farmers? *Environmental Health Perspectives* **111**(13): 1590-1594.

Hamscher, G., Sczesny, S., Hoper, H. and Nau, H. (2002). Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem* **74**(7): 1509-1518.

Hanon, J.-B., Jaspers, S., Butaye, P., Wattiau, P., Méroc, E., Aerts, M., Imberechts, H., Vermeersch, K. and Van der Stede, Y. (2015). A trend analysis of antimicrobial resistance in commensal *Escherichia coli* from several livestock species in Belgium (2011–2014). *Preventive Veterinary Medicine* **122**(4): 443-452.

Hawkey, P. M. and Jones, A. M. (2009). The changing epidemiology of resistance. *The Journal of Antimicrobial Chemotherapy* **64 Suppl 1**: i3-10.

Holman, D. B. and Chenier, M. R. (2013). Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. *FEMS Microbiology Ecology* **85**(1): 1-13.

Holman, D. B. and Chenier, M. R. (2014). Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. *FEMS Microbiology Ecology* **90**(3): 599-608.

Holmes, A. H., Moore, L. S. P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J. and Piddock, L. J. V. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet* **387**(10014): 176-187.

Honnorat-Benabbou, V. C., Lebugle, A. A., Sallek, B. and Duffaut-Lagarrigue, D. (2001). Stability study of tetracyclines with respect to their use in slow release systems. *Journal of Materials Science-Materials in Medicine* **12**(2): 107-110.

Hu, M. and Li, X. (2011) Barriers to Oral Bioavailability-An Overview. In *Oral Bioavailability: Basic Principles, Advanced Concepts, and Applications*. pp.1-7. Hoboken, New Jersey: John Wiley & Sons, Inc.

Hughes, D. and Brandis, G. (2013). Rifampicin Resistance: Fitness Costs and the Significance of Compensatory Evolution. *Antibiotics* **2**(2): 206-216.

Isaacson, R. and Kim, H. B. (2012). The intestinal microbiome of the pig. *Animal Health Research Reviews* **13**(1): 100-109.

Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjolund-Karlsson, M., Jansson, J. K. and Engstrand, L. (2010). Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *Plos One* **5**(3): e9836.

Jeters, R. T., Wang, G. R., Moon, K., Shoemaker, N. B. and Salyers, A. A. (2009). Tetracycline-associated transcriptional regulation of transfer genes of the *Bacteroides* conjugative transposon CTnDOT. *Journal of Bacteriology* **191**(20): 6374-6382.

JIACRA (2015). JIACRA. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-JIACRA-report.pdf.

Johnson, T. J., Shepard, S. M., Rivet, B., Danzeisen, J. L. and Carattoli, A. (2011). Comparative genomics and phylogeny of the IncI1 plasmids: A common plasmid type among porcine enterotoxigenic *Escherichia coli*. *Plasmid* **66**(3): 144-151.

Johnson, T. J., Singer, R. S., Isaacson, R. E., Danzeisen, J. L., Lang, K., Kobluk, K., Rivet, B., Borewicz, K., Frye, J. G., Englen, M., Anderson, J. and Davies, P. R. (2015). *In Vivo* Transmission of an IncA/C Plasmid in *Escherichia coli* Depends on Tetracycline Concentration, and Acquisition of the Plasmid Results in a Variable Cost of Fitness. *Applied and Environmental Microbiology* **81**(10): 3561-3570.

Johnston, C., Martin, B., Fichant, G., Polard, P. and Claverys, J.-P. (2014). Bacterial transformation: distribution, shared mechanisms and divergent control. *Nature Reviews Microbiology* **12**(3): 181-196.

Kalmokoff, M., Waddington, L. M., Thomas, M., Liang, K. L., Ma, C., Topp, E., Dandurand, U. D., Letellier, A., Matias, F. and Brooks, S. P. (2011). Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure. *Journal of Applied Microbiology* **110**(6): 1414-1425.

Kaushik, M., Kumar, S., Kapoor, R. K., Virdi, J. S. and Gulati, P. (2017). Integrons in Enterobacteriaceae: diversity, distribution and epidemiology. *International Journal of Antimicrobial Agents*, https://doi.org/10.1016/j.ijantimicag.2017.10.004.

Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J. and Isaacson, R. E. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary Microbiology* **153**(1-2): 124-133.

Kim, S., Yun, Z., Ha, U. H., Lee, S., Park, H., Kwon, E. E., Cho, Y., Choung, S., Oh, J., Medriano, C. A. and Chandran, K. (2014). Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative micro-contaminant concentrations. *Science of the Total Environment* **468-469**: 813-820.

Lamendella, R., Domingo, J. W., Ghosh, S., Martinson, J. and Oerther, D. B. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiology* **11**: 103.

Lancashire, J. F., Terry, T. D., Blackall, P. J. and Jennings, M. P. (2005). Plasmid-Encoded Tet B Tetracycline Resistance in Haemophilus parasuis. *Antimicrobial Agents and Chemotherapy* **49**(5): 1927-1931.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D. and Cars, O. (2013). Antibiotic resistance—the need for global solutions. *The Lancet Infectious Diseases* **13**(12): 1057-1098.

Leipe, D. D., Wolf, Y. I., Koonin, E. V. and Aravind, L. (2002). Classification and evolution of P-loop GTPases and related ATPases. *Journal of Molecular Biology* **317**(1): 41-72.

Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M. and Moller, K. (2002). Culture-Independent Analysis of Gut Bacteria: the Pig Gastrointestinal Tract Microbiota Revisited. *Applied and Environmental Microbiology* **68**(2): 673-690.

Leser, T. D., Lindecrona, R. H., Jensen, T. K., Jensen, B. B. and Møller, K. (2000). Changes in Bacterial Community Structure in the Colon of Pigs Fed Different Experimental Diets and after Infection with Brachyspira hyodysenteriae. *Applied and Environmental Microbiology* **66**(8): 3290-3296.

Leser, T. D. and Molbak, L. (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental Microbiology* **11**(9): 2194-2206.

Leverstein-van Hall, M. A., Box, A. T. A., Blok, H. E. M., Paauw, A., Fluit, A. C. and Verhoef, J. (2002). Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting. *Journal of Infectious Diseases* **186**(1): 49-56.

Levinson, W. (2014) Antimicrobial Drugs: Mechanism of Action. In *Review of Medical Microbiology and Immunology, 13e*. New York, NY: McGraw-Hill Education.

Levison, M. E. (2004). Pharmacodynamics of antimicrobial drugs. *Infectious Disease Clinics of North America* **18**(3): 451-465.

Levison, M. E. and Levison, J. H. (2009). Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. *Infectious Disease Clinics of North America* **23**(4): 791-vii.

Liu, A., Fong, A., Becket, E., Yuan, J., Tamae, C., Medrano, L., Maiz, M., Wahba, C., Lee, C., Lee, K., Tran, K. P., Yang, H. J., Hoffman, R. M., Salih, A. and Miller, J. H. (2011). Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: a Simple and Ultrasensitive Color Test for Detection of Antibiotics and Genotoxic Agents. *Antimicrobial Agents and Chemotherapy* **55**(3): 1204-1210.

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., Sul, W. J., Stedtfeld, T. M., Chai, B., Cole, J. R., Hashsham, S. A., Tiedje, J. M. and Stanton, T. B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences U.S.A.* **109**(5): 1691-1696.

Lopatkin, A. J., Huang, S., Smith, R. P., Srimani, J. K., Sysoeva, T. A., Bewick, S., Karig, D. K. and You, L. (2016). Antibiotics as a selective driver for conjugation dynamics. *Nature Microbiology* **1**(6): 16044.

Lorenz, M. G. and Wackernagel, W. (1994). Bacterial gene-transfer by natural genetic-transformation in the environment. *Microbiological Reviews* **58**: 563-602.

Lu, H.-Z., Weng, X.-H., Li, H., Yin, Y.-K., Pang, M.-Y. and Tang, Y.-W. (2002). *Enterococcus faecium*-Related Outbreak with Molecular Evidence of Transmission from Pigs to Humans. *Journal of Clinical Microbiology* **40**(3): 913-917.

Lundstrom, S. V., Ostman, M., Bengtsson-Palme, J., Rutgersson, C., Thoudal, M., Sircar, T., Blanck, H., Eriksson, K. M., Tysklind, M., Flach, C.-F. and Larsson, D. G. J. (2016). Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. *Science of the Total Environment* **553**: 587-595.

Luo, X., Zheng, Z., Greaves, J., Cooper, W. J. and Song, W. (2012). Trimethoprim: Kinetic and mechanistic considerations in photochemical environmental fate and AOP treatment. *Water Research* **46**(4): 1327-1336.

Mach, N., Berri, M., Estelle, J., Levenez, F., Lemonnier, G., Denis, C., Leplat, J. J., Chevaleyre, C., Billon, Y., Dore, J., Rogel-Gaillard, C. and Lepage, P. (2015). Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environmental Microbiology Reports* **7**(3): 554-569.

Maguire, H. C., Codd, A. A., Mackay, V. E., Rowe, B. and Mitchell, E. (1993). A large outbreak of human salmonellosis traced to a local pig farm. *Epidemiology and Infection* **110**(2): 239-246.

Maiques, E., Ubeda, C., Campoy, S., Salvador, N., Lasa, I., Novick, R. P., Barbe, J. and Penades, J. R. (2006). beta-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in Staphylococcus aureus. *Journal of Bacteriology* **188**(7): 2726-2729.

Mandell, L. A., Ball, P. and Tillotson, G. (2001). Antimicrobial Safety and Tolerability: Differences and Dilemmas. *Clinical Infectious Diseases* **32**(Supplement_1): S72-S79.

Mao, E. F., Lane, L., Lee, J. and Miller, J. H. (1997). Proliferation of mutators in A cell population. *Journal of Bacteriology* **179**(2): 417-422.

MARAN (2017). Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands. http://www.wur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/Bioveterinary-Research/Publicaties/MARAN-Rapporten.htm.

Marchant, M., Vinue, L., Torres, C. and Moreno, M. A. (2013). Change of integrons over time in *Escherichia coli* isolates recovered from healthy pigs and chickens. *Veterinary Microbiology* **163**(1-2): 124-132.

Marshall, B. M. and Levy, S. B. (2011). Food Animals and Antimicrobials: Impacts on Human Health. *Clinical Microbiology Reviews* **24**(4): 718-733.

Marti, E., Variatza, E. and Balcazar, J. L. (2014). Bacteriophages as a reservoir of extended-spectrum beta-lactamase and fluoroquinolone resistance genes in the environment. *Clinical Microbiology and Infection* **20**(7): 456-459.

Martinez, J. L. (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society B-Biological Sciences* **276**(1667): 2521-2530.

Martinez, M. and Modric, S. (2010). Patient variation in veterinary medicine: part I. Influence of altered physiological states. *Journal of Veterinary Pharmacology and Therapeutics* **33**(3): 213-226.

Martinez, M. N. (1998a). Special series - Use of pharmacokinetics in veterinary medicine - Article II: Volume, clearance, and half-life. *Journal of the American Veterinary Medical Association* **213**(8): 1122-1127.

Martinez, M. N. (1998b). Use of pharmacokinetics in veterinary medicine - Article III: Physicochemical properties of pharmaceuticals. *Journal of the American Veterinary Medical Association* **213**(9): 1274-1277.

Martinez, M. N., Toutain, P. L. and Turnidge, J. (2013) Antimicrobial Therapy in Veterinary Medicine. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.79-103. Hoboken, NJ: John Wiley & Sons, Inc.

Mathur, S. and Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria--a review. *International Journal of Food Microbiology* **105**(3): 281-295.

McEvoy, J. D. G. (2002). Contamination of animal feedingstuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. *Analytica Chimica Acta* **473**(1-2): 3-26.

McEwen, S. A. and Fedorka-Cray, P. J. (2002). Antimicrobial use and resistance in animals. *Clinical Infectious Diseases* **34**: S93-S106.

Messens, W., Goris, J., Dierick, N., Herman, L. and Heyndrickx, M. (2010). Inhibition of *Salmonella* Typhimurium by medium-chain fatty acids in an in vitro simulation of the porcine cecum. *Veterinary Microbiology* **141**(1-2): 73-80.

Modi, S. R., Lee, H. H., Spina, C. S. and Collins, J. J. (2013). Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* **499**(7457): 219-222.

Molbak, K., Baggesen, D. L., Aarestrup, F. M., Ebbesen, J. M., Engberg, J., Frydendahl, K., Gerner-Smidt, P., Petersen, A. M. and Wegener, H. C. (1999). An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. *The New England Journal of Medicine* **341**(19): 1420-1425.

Moller, T. S. B., Overgaard, M., Nielsen, S. S., Bortolaia, V., Sommer, M. O. A., Guardabassi, L. and Olsen, J. E. (2016). Relation between *tetR* and *tetA* expression in tetracycline resistant *Escherichia coli*. *BMC Microbiology* **16**.

Moon, K., Shoemaker, N. B., Gardner, J. F. and Salyers, A. A. (2005). Regulation of excision genes of the *Bacteroides* conjugative transposon CTnDOT. *Journal of Bacteriology* **187**(16): 5732-5741.

Moore, I. F., Hughes, D. W. and Wright, G. D. (2005). Tigecycline is modified by the flavin-dependent monooxygenase TetX. *Biochemistry* **44**(35): 11829-11835.

Muniesa, M., Colomer-Lluch, M. and Jofre, J. (2013). Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. *Future Microbiology* **8**(6): 739-751.

Munita, J. M. and Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology spectrum* **4**(2): 10.1128/microbiolspec.VMBF-0016-2015.

Ng, L. K., Martin, I., Alfa, M. and Mulvey, M. (2001). Multiplex PCR for the detection of tetracycline resistant genes. *Molecular and Cellular Probes* **15**(4): 209-215.

Nguyen, F., Starosta, A. L., Arenz, S., Sohmen, D., Donhofer, A. and Wilson, D. N. (2014). Tetracycline antibiotics and resistance mechanisms. *Biological Chemistry* **395**(5): 559-575.

Nielsen, K. M., Johnsen, P. J., Bensasson, D. and Daffonchio, D. (2007). Release and persistence of extracellular DNA in the environment. *Environmental Biosafety Research* **6**(1-2): 37-53.

Nielsen, P. and Gyrd-Hansen, N. (1994). Oral bioavailability of sulphadiazine and trimethoprim in fed and fasted pigs. *Research in Veterinary Science* **56**(1): 48-52.

Nielsen, P. and GyrdHansen, N. (1996). Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs. *Journal of Veterinary Pharmacology and Therapeutics* **19**(4): 305-311.

Nocker, A., Burr, M. and Camper, A. K. (2007). Genotypic microbial community profiling: a critical technical review. *Microbial Ecology* **54**(2): 276-289.

Nonaka, L., Connell, S. R. and Taylor, D. E. (2005). 16S rRNA mutations that confer tetracycline resistance in *Helicobacter pylori* decrease drug binding in *Escherichia coli* ribosomes. *Journal of Bacteriology* **187**(11): 3708-3712.

NORM-VET (2017). Report on Usage of Antimicrobial Agents and Occurence of Antimicrobial Resistance in Norway in animals and humans. http://wwweng.vetinst.no/eng/Publications/NORM-NORM-VET-Report.html.

OIE (2011). Terrestrial Animal Health Code. http://web.oie.int/eng/normes/mcode/en_glossaire.htm#terme_antibiotique.

OIE (2015). World Organisation for Animal Health. OIE list of antimicrobial agents of veterinary importance. http://www.oie.int/our-scientific-expertise/veterinary-products/antimicrobials/.

OIE (2016). World Organization for Animal Health. The OIE Strategy on Antimicrobial Resistance and the Prudent Use of Antimicrobials. www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/PortailAMR/EN_OIE-AMRstrategy.pdf.

Olofsson, S. K. and Cars, O. (2007). Optimizing drug exposure to minimize selection of antibiotic resistance. *Clinical Infectious Diseases* **45**: S129-S136.

Page, S. W. and Gautier, P. (2012). Use of antimicrobial agents in livestock. *Revue Scientifique Et Technique-Office International Des Epizooties* **31**(1): 145-188.

Pakpour, S., Jabaji, S. and Chenier, M. R. (2012). Frequency of antibiotic resistance in a swine facility 2.5 years after a ban on antibiotics. *Microbial Ecology* **63**(1): 41-50.

Park, S.-J., Kim, J., Lee, J.-S., Rhee, S.-K. and Kim, H. (2014). Characterization of the fecal microbiome in different swine groups by high-throughput sequencing. *Anaerobe* **28**: 157-162.

Pereira, R. V., Siler, J. D., Bicalho, R. C. and Warnick, L. D. (2014). *In vivo* selection of resistant *E. coli* after ingestion of milk with added drug residues. *Plos One* **9**(12): e115223.

PHYSPROP (2017). Physical properties database. SRC. https://www.srcinc.com/what-we-do/environmental/scientific-databases.html.

Pijpers, A., Schoevers, E. J., van Gogh, H., van Leengoed, L. A., Visser, I. J., van Miert, A. S. and Verheijden, J. H. (1991). The influence of disease on feed and water consumption and on pharmacokinetics of orally administered oxytetracycline in pigs. *Journal of Animal Science* **69**(7): 2947-2954.

Prescott, J. F. (2013) Sulfonamides, Diaminopyrimidines and Their Combinations. In *Antimicrobial Therapy in Veterinary Medicine* eds. Giguère, S., Prescott, J. F. and Dowling, P. M. pp.279-294. Hoboken, NJ: John Wiley & Sons, Inc.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. and Claverys, J. P. (2006). Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science* **313**(5783): 89-92.

Prüller, S., Rensch, U., Meemken, D., Kaspar, H., Kopp, P. A., Klein, G. and Kehrenberg, C. (2015). Antimicrobial Susceptibility of Bordetella bronchiseptica Isolates from Swine and Companion Animals and Detection of Resistance Genes. *Plos One* **10**(8): e0135703.

Putier, F. (2010). Evaluation of cross-contamination in livestock feed delivery trucks. Technics. *Feed magazine/Kraftfutter*: 9-10.

Qiang, Z. and Adams, C. (2004). Potentiometric determination of acid dissociation constants (pKa) for human and veterinary antibiotics. *Water Research* **38**(12): 2874-2890.

Quiroga, M. P., Andres, P., Petroni, A., Soler Bistué, A. J. C., Guerriero, L., Vargas, L. J., Zorreguieta, A., Tokumoto, M., Quiroga, C., Tolmasky, M. E., Galas, M. and Centrón, D. (2007). Complex Class 1 Integrons with Diverse Variable Regions, Including aac(6)-Ib-cr, and a Novel Allele, qnrB10, Associated with ISCR1 in Clinical Enterobacterial Isolates from Argentina. *Antimicrobial Agents and Chemotherapy* **51**(12): 4466-4470.

Rescigno, A. (1997). Fundamental concepts in pharmacokinetics. *Pharmacological Research* **35**(5): 363-390.

Rettedal, E., Vilain, S., Lindblom, S., Lehnert, K., Scofield, C., George, S., Clay, S., Kaushik, R. S., Rosa, A. J., Francis, D. and Brozel, V. S. (2009). Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline. *Applied and Environmental Microbiology* **75**(17): 5489-5495.

Reyns, T., De Boever, S., Baert, K., Croubels, S., Schauvliege, S., Gasthuys, F. and De Backer, P. (2007). Disposition and oral bioavailability of amoxicillin and clavulanic acid in pigs. *Journal of Veterinary Pharmacology and Therapeutics* **30**(6): 550-555.

Roberts, M. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Reviews* **19**(1): 1-24.

Roberts, M. (2016). Mechanism of resistance for characterized *tet* and *otr* genes. https://faculty.washington.edu/marilynr/tetweb1.pdf.

Royal Decree (2016). Koninklijk Besluit van 21/07/2016 betreffende de voorwaarden voor het gebruik van geneesmiddelen door de dierenartsen en door de verantwoordelijken van de dieren. www.afsca.be/dierlijkeproductie/dieren/diergeneesmiddelen/_documents/2016_07_21_KB21juli2016_AR21juillet2016_BS_MB.pdf.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy* **51**(5): 1109-1117.

Sabaté, M., Navarro, F., Miró, E., Campoy, S., Mirelis, B., Barbé, J. and Prats, G. (2002). Novel Complex *sul*1-Type Integron in *Escherichia coli* Carrying *bla*(CTX-M-9). *Antimicrobial Agents and Chemotherapy* **46**(8): 2656-2661.

Saenger, W., Orth, P., Kisker, C., Hillen, W. and Hinrichs, W. (2000). The Tetracycline Repressor-A Paradigm for a Biological Switch. *Angewandte Chemie International Edition* **39**(12): 2042-2052.

Salyers, A. A. (1993). Gene transfer in the mammalian intestinal tract. *Current Opinion in Biotechnology* **4**(3): 294-298.

Salyers, A. A., Shoemaker, N. B., Stevens, A. M. and Li, L. Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews* **59**(4): 579-590.

Sanchez-Pescador, R., Brown, J. T., Roberts, M. and Urdea, M. S. (1988). Homology of the TetM with translational elongation factors: implications for potential modes of *tet*M-conferred tetracycline resistance. *Nucleic Acids Research* **16**(3): 1218.

Sandegren, L. (2014). Selection of antibiotic resistance at very low antibiotic concentrations. *Upsala Journal of Medical Sciences* **119**(2): 103-107.

Sanders, P., Gicquel, M., Hurtaud, D., Chapel, A. M., Gaudiche, C. and Bousquet, E. (1996). Absolute bioavailability of doxycycline after oral administration in medicated feed to pigs. Proceedings of the 14th IPVS Congress. Bologna, Italy**:** 663.

Sanderson, H., Fricker, C., Brown, R. S., Majury, A. and Liss, S. N. (2016). Antibiotic resistance genes as an emerging environmental contaminant. *Environmental Reviews* **24**(2): 205-218.

Sandoval-Motta, S. and Aldana, M. (2016). Adaptive resistance to antibiotics in bacteria: a systems biology perspective. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* **8**(3): 253-267.

Santos, M. D. F., Vermeersch, H., Remon, J. P., Schelkens, M., DeBacker, H. J. J., VanBree, H. J. J., Ducatelle, R. and Haesebrouck, F. (1996). Pharmacokinetics and bioavailability of doxycycline in turkeys. *Journal of Veterinary Pharmacology and Therapeutics* **19**(4): 274-280.

Schouten, E. G. (2011). Bureau Risicobeoordeling en onderzoeksprogrammering. Advies over toediening van antibiotica in de veehouderij. https://www.nvwa.nl/onderwerpen/antibiotica-in-de-

veehouderij/documenten/risicobeoordeling/voedselveiligheid/archief/2016m/antibiotica-in-de-veehouderij.

Schwarz, S. and Chaslus-Dancla, E. (2001a). Use of antimicrobials in veterinary medicine and mechanisms of resistance. *Veterinary Research* **32**(3-4): 201-225.

Schwarz, S., Kehrenberg, C. and Walsh, T. R. (2001b). Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents* **17**(6): 431-437.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**(14): 2068-2069.

Segato, G., Benetti, C., Angeletti, R., Montesissa, C. and Biancotto, G. (2011). Doxycycline and sulfadimethoxine transfer from cross-contaminated feed to chicken tissues. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **28**(7): 860-868.

Seputiene, V., Bogdaite, A., Ruzauskas, M. and Suziedeliene, E. (2012). Antibiotic resistance genes and virulence factors in Enterococcus faecium and Enterococcus faecalis from diseased farm animals: pigs, cattle and poultry. *Pol J Vet Sci* **15**(3): 431-438.

Shentu, J.-l., Zhang, K., Shen, D.-s., Wang, M.-z. and Feng, H.-j. (2015). Effect from low-level exposure of oxytetracycline on abundance of tetracycline resistance genes in arable soils. *Environmental Science and Pollution Research* **22**(17): 13102-13110.

Sherratt, D. J. (1982). The maintenance and propagation of plasmid genes in bacterial populations. The Sixth Fleming Lecture. *Journal of General Microbiology* **128**(4): 655-661.

Showsh, S. A. and Andrews Jr, R. E. (1992). Tetracycline enhances Tn916-mediated conjugal transfer. *Plasmid* **28**(3): 213-224.

Siguier, P., Filee, J. and Chandler, M. (2006). Insertion sequences in prokaryotic genomes. *Current Opinion in Microbiology* **9**(5): 526-531.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., Haesebrouck, F. and Butaye, P. (2010). Broad-spectrum beta-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. *FEMS Microbiology Reviews* **34**(3): 295-316.

Smet, A., Rasschaert, G., Martel, A., Persoons, D., Dewulf, J., Butaye, P., Catry, B., Haesebrouck, F., Herman, L. and Heyndrickx, M. (2011). *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology* **110**(2): 541-549.

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C. and de la Cruz, F. (2010). Mobility of Plasmids. *Microbiology and Molecular Biology Reviews* **74**(3): 434-452.

Sommer, F. and Backhed, F. (2013). The gut microbiota--masters of host development and physiology. *Nature Reviews Microbiology* **11**(4): 227-238.

Sorensen, A. C., Lawrence, R. S. and Davis, M. F. (2014). Interplay between policy and science regarding low-dose antimicrobial use in livestock. *Frontiers in Microbiology* **5**.

Speer, B. S. and Salyers, A. A. (1989). Novel aerobic tetracycline resistance gene that chemically modifies tetracycline. *Journal of Bacteriology* **171**(1): 148-153.

Spencer, S. J., Tamminen, M. V., Preheim, S. P., Guo, M. T., Briggs, A. W., Brito, I. L., A Weitz, D., Pitkänen, L. K., Vigneault, F., Virta, M. P. and Alm, E. J. (2016). Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. *The ISME Journal* **10**(2): 427-436.

Stephens, C. R., Conover, L. H., Hochstein, F. A., Regna, P. P., Pilgrim, F. J., Brunings, K. J. and Woodward, R. B. (1952). Terramycin. VIII. Structure of Aureomycin and Terramycin. *Journal of the American Chemical Society* **74**(19): 4976-4977.

Stevens, A. M., Shoemaker, N. B., Li, L. Y. and Salyers, A. A. (1993). Tetracycline regulation of genes on *Bacteroides* conjugative transposons. *Journal of Bacteriology* **175**(19): 6134-6141.

Stolker, A. A. M., Manti, V., Zuidema, T., van Egmond, H., Deckers, E. R., Herbes, R., Hooglugt, J., Heuvel, E. O. and de Jong, J. (2013). Carry-over of veterinary drugs from medicated to non-medicated feeds in commercial feed manufacturing plants. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **30**(6): 1100-1107.

Sum, P. E., Lee, V. J., Testa, R. T., Hlavka, J. J., Ellestad, G. A., Bloom, J. D., Gluzman, Y. and Tally, F. P. (1994). Glycylcyclines. 1. A new generation of potent antibacterial agents through modification of 9-aminotetracyclines. *Journal of Medicinal Chemistry* **37**(1): 184-188.

Swedres-Svarm (2017). Swedres-Svarm reports. Swedish Veterinary Antimicrobial Resistance Monitoring. http://www.sva.se/en/antibiotics/svarm-reports.

Thaker, M., Spanogiannopoulos, P. and Wright, G. D. (2010). The tetracycline resistome. *Cellular and Molecular Life Sciences* **67**(3): 419-431.

Thomas, C. M. and Nielsen, K. M. (2005). Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria. *Nature Reviews Microbiology* **3**(9): 711-721.

Timmerman, T., Dewulf, J., Catry, B., Feyen, B., Opsomer, G., de Kruif, A. and Maes, D. (2006). Quantification and evaluation of antimicrobial drug use in group treatments for fattening pigs in Belgium. *Preventive Veterinary Medicine* **74**(4): 251-263.

Toleman, M. A., Bennett, P. M. and Walsh, T. R. (2006). ISCR elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews* **70**(2): 296-316.

Torres, O. R., Korman, R. Z., Zahler, S. A. and Dunny, G. M. (1991). The conjugative transposon Tn925: enhancement of conjugal transfer by tetracycline in *Enterococcus faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E. faecalis*. *Molecular and General Genetics* **225**(3): 395-400.

Tupin, A., Gualtieri, M., Roquet-Baneres, F., Morichaud, Z., Brodolin, K. and Leonetti, J. P. (2010). Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *International Journal of Antimicrobial Agents* **35**(6): 519-523.

Valentine, P. J., Shoemaker, N. B. and Salyers, A. A. (1988). Mobilization of *Bacteroides* plasmids by *Bacteroides* conjugal elements. *Journal of Bacteriology* **170**(3): 1319-1324.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A. and Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences* **112**(18): 5649-5654.

van Krimpen, M. M. (2007). Medicijnverstrekking aan groepen varkens. *Animal Science Group, Wageningen UR*.

Van Meervenne, E., Van Coillie, E., Kerckhof, F. M., Devlieghere, F., Herman, L., De Gelder, L. S. P., Top, E. M. and Boon, N. (2012). Strain-Specific Transfer of Antibiotic Resistance from an Environmental Plasmid to Foodborne Pathogens. *Journal of Biomedicine and Biotechnology*, 10.1155/2012/834598: 8.

Vandenberge, V., Delezie, E., Huyghebaert, G., Delahaut, P., Daeseleire, E. and Croubels, S. (2012a). Residues of sulfadiazine and doxycycline in broiler liver and muscle tissue due to cross-contamination of feed. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **29**(2): 180-188.

Vandenberge, V., Delezie, E., Huyghebaert, G., Delahaut, P., De Backer, P., Daeseleire, E. and Croubels, S. (2012b). Residues of sulfadiazine and doxycycline in egg matrices due to cross-contamination in the feed of laying hens and the possible correlation with physicochemical, pharmacokinetic and physiological parameters. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment* **29**(6): 908-917.

Versalovic, J., Koeuth, T. and Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**(24): 6823-6831.

VICH_GL_49. Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food producing animals: validation of analytical methods used in residue depletion studies. http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM207942.pdf.

Viljanen, P. and Boratynski, J. (1991). The susceptibility of conjugative resistance transfer in Gram-negative bacteria to physicochemical and biochemical agents. *FEMS Microbiology Letters* **88**(1): 43-54.

Vogwill, T. and MacLean, R. C. (2015). The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evolutionary Applications* **8**(3): 284-295.

Volkers, G., Palm, G. J., Weiss, M. S., Wright, G. D. and Hinrichs, W. (2011). Structural basis for a new tetracycline resistance mechanism relying on the TetX monooxygenase. *FEBS Letters* **585**(7): 1061-1066.

Vukmirović, D., Duragić, O., Sredanović, S., Lević, J., Čolović, R., Ivanov, D. and Kokić, B. (2010). Determination of working accuracy as an important step for implementation of corrective measures in feed plant. . *Food & Feed Research* **37**: 7-12.

Wang, J., Stephan, R., Power, K., Yan, Q., Hachler, H. and Fanning, S. (2014). Nucleotide sequences of 16 transmissible plasmids identified in nine multidrug-

resistant *Escherichia coli* isolates expressing an ESBL phenotype isolated from food-producing animals and healthy humans. *The Journal of Antimicrobial Chemotherapy* **69**(10): 2658-2668.

Weisser, J. and Wiedemann, B. (1987). Inhibition of R-plasmid transfer in *Escherichia coli* by 4-quinolones. *Antimicrobial Agents and Chemotherapy* **31**(4): 531-534.

Whittle, G., Shoemaker, N. B. and Salyers, A. A. (2002). Characterization of genes involved in modulation of conjugal transfer of the *Bacteroides* conjugative transposon CTnDOT. *Journal of Bacteriology* **184**(14): 3839-3847.

WHO (2000). World Health Organisation. Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food. http://www.who.int/foodsafety/publications/containment-amr/en/.

WHO (2015). Global Action Plan on Antimicrobial Resistance. http://www.who.int/antimicrobial-resistance/global-action-plan/en/.

WHO (2016). World Health Organization. Critically Important Antimicrobials for Human Medicine. Fourth revision 2013. Bogota, Colombia, 2013. http://www.who.int/foodsafety/publications/antimicrobials-fourth/en/.

Witt, J. (2006). Pharmacokinetics of sulfadiazine in pigs, Osnabrück University.

Wolkow, C. A., DeBoy, R. T. and Craig, N. L. (1996). Conjugating plasmids are preferred targets for Tn7. *Genes & Development* **10**(17): 2145-2157.

Wu, Y. and Fassihi, R. (2005). Stability of metronidazole, tetracycline HCl and famotidine alone and in combination. *International Journal of Pharmaceutics* **290**(1): 1-13.

Yang, H., Huang, X. C., Fang, S. M., Xin, W. S., Huang, L. S. and Chen, C. Y. (2016). Uncovering the composition of microbial community structure and metagenomics among three gut locations in pigs with distinct fatness. *Scientific Reports* **6**.

Yang, W., Moore, I. F., Koteva, K. P., Bareich, D. C., Hughes, D. W. and Wright, G. D. (2004). TetX is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. *Journal of Biological Chemistry* **279**(50): 52346-52352.

Yates, J. W. T. and Arundel, P. A. (2008). On the volume of distribution at steady state and its relationship with two-compartmental models. *Journal of Pharmaceutical Sciences* **97**(1): 111-122.

Zessel, K., Mohring, S., Hamscher, G., Kietzmann, M. and Stahl, J. (2014). Biocompatibility and antibacterial activity of photolytic products of sulfonamides. *Chemosphere* **100**: 167-174.

Zhang, J. M., Yang, X. W., Kuang, D., Shi, X. M., Xiao, W. J., Zhang, J., Gu, Z., Xu, X. B. and Meng, J. H. (2015). Prevalence of antimicrobial resistance of non-typhoidal *Salmonella* serovars in retail aquaculture products. *International Journal of Food Microbiology* **210**: 47-52.

Zhang, P. Y., Xu, P. P., Xia, Z. J., Wang, J., Xiong, J. and Li, Y. Z. (2013). Combined treatment with the antibiotics kanamycin and streptomycin promotes the conjugation of *Escherichia coli*. *FEMS Microbiology Letters* **348**(2): 149-156.

Zhang, X., McDaniel, A. D., Wolf, L. E., Keusch, G. T., Waldor, M. K. and Acheson, D. W. (2000). Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *Journal of Infectious Diseases* **181**(2): 664-670.

Zuidema, T., Stolker, A. A. M. and Van Ginkel L., A. (2014). Kruiscontaminatie van antibiotica. Onderzoek naar de aanwezigheid van antibioticaresiduen in mest van vleesvarkens en vleeskalveren en op pluimveebedrijven. Wageningen, RIKILT Wageningen UR (University and Research centre)**:** 48.

CURRICULUM VITÆ

Laura Peeters werd geboren op 9 november 1985 te Eeklo. In 2003 behaalde ze het diploma secundair onderwijs richting Wetenschappen-Wiskunde (8u) aan het College O.-L.-V. Ten Doorn te Eeklo. In hetzelfde jaar startte ze de studie Diergeneeskunde aan de Universiteit Gent en in 2009 behaalde ze het diploma van Master in de Diergeneeskunde (optie Kleine Huisdieren).

Na een aantal jaren in een dierenartsenpraktijk gewerkt te hebben, startte ze haar doctoraatsonderzoek naar de effecten van kruiscontaminatie van diervoeder met antimicrobiële middelen op resistentieselectie, gefinancierd door de FOD Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. Het onderzoek werd uitgevoerd op het CODA in Ukkel, de Faculteit Diergeneeskunde (Vakgroep Farmacologie, Toxicologie en Biochemie en Vakgroep Pathologie, Bacteriologie en Pluimveeziekten) in Merelbeke en op het ILVO (Eenheid Technologie en Voeding) in Melle.

Ze begeleidde studenten in het behalen van hun masterproef en in 2015 vervolledigde ze de doctoraatsopleiding van de Doctoral School of Life Science and Medicine van de Universiteit Gent.

Laura Peeters is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en nam actief deel aan verschillende nationale en internationale congressen aan de hand van posters en mondelinge presentaties.

BIBLIOGRAPHY

1 PUBLICATIONS IN INTERNATIONAL JOURNALS

— **Peeters, L. E. J.**, De Mulder, T., Van Coillie, E., Huygens, J., Smet, A., Daeseleire, E., Dewulf, J., Imberechts, H., Butaye, P., Haesebrouck, F., Croubels, S., Heyndrickx, M. and Rasschaert, G. (2017). Selection and transfer of an IncI1-tet(A) plasmid of Escherichia coli in an ex vivo model of the porcine caecum at doxycycline concentrations caused by cross-contaminated feed. Journal of Applied Microbiology **123**(5): 1312-1320.

— **Peeters, L. E. J.**, Croubels, S., Rasschaert, G., Imberechts, H., Daeseleire, E., Dewulf, J., Heyndrickx, M., Butaye, P., Haesebrouck, F. and Smet, A. (2018). Effect of residual doxycycline concentrations on resistance selection and transfer in porcine commensal Escherichia coli. International Journal of Antimicrobial Agents, 51(1):123-127.

— **Peeters, L. E.**, Daeseleire, E., Devreese, M., Rasschaert, G., Smet, A., Dewulf, J., Heyndrickx, M., Imberechts, H., Haesebrouck, F., Butaye, P. and Croubels, S. (2016). Residues of chlortetracycline, doxycycline and sulphadiazine-trimethoprim in intestinal content and feces of pigs due to cross-contamination of feed. BMC Veterinary Research **12**: 209.

— **Peeters, L. E. J.**, Argudín, M. A., Azadikhah, S. and Butaye, P. (2015). Antimicrobial resistance and population structure of *Staphylococcus aureus* recovered from pigs farms. Veterinary Microbiology **180**(1–2): 151-156.

— Oosterik, L. H., **Peeters, L.**, Mutuku, I., Goddeeris, B. M. and Butaye, P. (2014). Susceptibility of avian pathogenic *Escherichia coli* from laying hens in Belgium to antibiotics and disinfectants and integron prevalence. Avian Diseases **58**(2): 271-278.

2 CONTRIBUTIONS AT NATIONAL AND INTERNATIONAL CONFERENCES

— **Peeters, L.**, Antimicrobiële resistentie - Impact van kruisbesmetting van diervoeders met antimicrobiële middelen op de resistentieontwikkeling.
Symposium Contractueel Onderzoek 'Voedselveiligheid van A(ntibiotica) tot Z(earalenone), Brussels, Belgium, October 25th-26th 2017

— **Peeters, L.**, *Kruiscontaminatie van varkensvoeder met antibiotica en mogelijke effecten op resistentieselectie en –transfer.*
BAPCOC Symposium 'Voorbeelden van goede praktijken om antibioticaresistentie te verminderen' Brussels, Belgium, November 18th 2016

— **Peeters, L.**, Rasschaert, G., Smet, A., Butaye, P., Croubels, S., Imberechts, H., Daeseleire, E., Dewulf, J., Haesebrouck, F., and Heyndrickx, M., *Transfer of a tetracycline resistance gene in the simulated pig caecum at doxycycline concentrations caused by cross-contaminated feed.*
Belgian Society for Food Microbiology, Twenty-first Conference on Food Microbiology. Brussels, Belgium, September 15th — 16th 2016

— **Peeters, L.**, Argudín, M.A., Azadikhah S., Butaye, P., *Antimicrobial resistance and population structure of methicillin-resistant* Staphylococcus aureus *recovered from pigs.*
4th ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, Washington DC, US, May 8th — 11th 2015

— **Peeters, L.**, Argudín, M.A., Daeseleire, E. , Dewulf, J., Heyndrickx, M., Haesebrouck, F., Croubels, S., Butaye, P., *Tetracycline, sulfonamide and trimethoprim resistance genes and integrons in* Escherichia coli.
SfAM/MVNA Summer Conference 2014, Brighton, UK, June 30th — July 3rd 2014

— **Peeters, L.**, Daeseleire, E. , Devreese, M., Dewulf, J., Heyndrickx, M., Haesebrouck, F., Butaye, P., Croubels, S., Concentrations of selected antimicrobials in caecum, colon and manure of pigs due to a 3% cross-contamination of the feed.
Seventh International Symposium on Hormone and Veterinary Drug Residue Analysis, Ghent, Belgium, June 2nd — June 5th 2014

— **Peeters, L.**, Argudín, M.A., Butaye, P., Antimicrobial resistance in E. coli isolated from food producing animals.
5th Symposium on Antimicrobial Resistance in Animals and the Environment, Ghent, Belgium, June 30th — July 3rd 2013

— **Peeters, L.**, Argudín, M.A., Butaye, P., Antimicrobial resistance in E. faecium and E. faecalis isolated from poultry and bovines.
5th Symposium on Antimicrobial Resistance in Animals and the Environment, Ghent, Belgium, June 30th — July 3rd 2013

— **Peeters, L.**, Argudín, M.A., Butaye, P., Antimicrobial resistance in E. hirae isolated from food producing animals.
5th Symposium on Antimicrobial Resistance in Animals and the Environment, Ghent, Belgium, June 30th — July 3rd 2013

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