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ORIGINAL ARTICLE

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

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Abstract

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

Methods and Results: 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^{-1} 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^{-1} 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^{-1} DOX compared to medium without DOX.

Conclusions: 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.

Significance and Impact of the Study: 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.

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 and Hughes 2014), however, the extent of this has not been quantified. Pig feed may become contaminated with antimicrobials through carry-over from medicated to nonmedicated feed at the feed mill, during transport or

at the farm (Stolker *et al.* 2013; Filippitzi *et al.* 2016). 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 Denmark and the Netherlands, consequently cross-contamination at the feed mill is ruled out in these countries. 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 doxycycline (DOX), chlortetracycline or sulphadiazine–trimethoprim in pigs' intestines, due to a 3% carry-over level in the feed, have been determined before in an *in vivo* study (Peeters *et al.* 2016). 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^{-1} 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^{-1}). As a consequence of a recent study by Belgian covenant (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^{-1} DOX).

The selective pressure of these two concentrations of DOX has recently been investigated using pure bacterial cultures (Peeters *et al.* 2017). These competition studies between DOX-resistant and -susceptible porcine commensal *Escherichia coli* strains showed that both 1 and 4 mg l^{-1} 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 coselected and/or be cotransferred (Leverstein-van Hall *et al.* 2002; Gullberg *et al.* 2014).

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 (Peeters *et al.* 2017) 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.

Materials and methods

Bacterial strain

EC 682 is a commensal *E. coli* strain that was isolated from pig faeces during a national Belgian antimicrobial resistance monitoring programme (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 (Peeters *et al.* 2017). A nonlactose-fermenting mutant of this strain was selected to be able to distinguish it from other (lactose fermenting) *E. coli* on MacConkey no. 3 agar, on which this mutant forms white colonies (Smet *et al.* 2011). The nonlactose-fermenting mutant showed the same minimum inhibitory concentration (MIC) for DOX as the original strain, namely 16 mg l^{-1}. The *in vitro* growth rate of this strain (0·245 min^{-1}) was not affected by the presence of 1 mg l^{-1} DOX, whereas 4 mg l^{-1} DOX reduced the growth rate slightly by 0·0037 min^{-1}. The transfer frequency (ratio transconjugants/total recipients after 24 h incubation) of pEC682 to two different recipient strains ($1{\cdot}58 \times 10^{-5}$ and $1{\cdot}57 \times 10^{-6}$ respectively) was not affected significantly by the presence of 1 mg l^{-1} DOX or 4 mg l^{-1} DOX (Peeters *et al.* 2017).

Caecal 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) and a 1·3-l reactor vessel. At day 0, the reactor vessel was filled with 0·5 l of nutritional medium (Table 1) 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 rev min^{-1}) were maintained and the headspace of the vessel was flushed constantly with a 80% nitrogen–20% carbon dioxide mixture at 20 ml min^{-1} to create anaerobic conditions. 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 per 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 for 24 h (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^{-1} 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^{-1} NaOH solution. At day 3, the nutritional medium was supplemented with 1 or 4 mg l^{-1} DOX (doxycycline hyclate; Fagron, Waregem, Belgium) and continuously administered until the end of the experiment, that is, day 9. For each condition (0, 1 and 4 mg l^{-1} 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.

Table 1 Composition of the nutritional medium

	g l^{-1}
Starch from corn*	5
Casein from bovine milk*	10
Casein hydrolysate acid*	0·5
Soybean oil (Carrefour sojaolie)§	1
L-cysteine hydrochloride anhydrous*	0·65
Pectin from citrus peel*	2·7
Alphacel†	13·8
Mucin from porcine stomach, type II*	5
Vitamin–mineral premix¶	2·35
KH_2PO_4‡	0·93
Na_2HPO_4 $12H_2O$*	1·12

*Sigma-Aldrich, Bornem, Belgium.
†MP Biomedicals, Brussels, Belgium.
‡Merck, Overijse, Belgium.
§N.V. Carrefour, Evere, Belgium.
¶Vitamex N.V., Drongen, Belgium.
The medium was acidified to pH 2 with 4 ml l^{-1} HCl 37% (Merck).

Bacterial population dynamics

Tenfold serial dilutions of reactor content samples were plated daily in duplicate on MacConkey no. 3 agar (MC) (Oxoid, Basingstoke, UK) with or without 8 mg l^{-1} DOX (= maximum cut-off value of DOX considering coliform bacteria according to EUCAST (2016) data) and incubated overnight at 37°C. Samples of the negative control run without EC 682 were plated on MC, MC + 4 mg l^{-1} DOX (=EUCAST ECOFF DOX for *E. coli*) and MC + 8 mg l^{-1} 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 for 48 h at 37°C and the total number of colonies was counted.

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^{-1} 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^{-1} 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^{-1} 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 (Ng *et al.* 2001; Carattoli *et al.* 2005) 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 mmol l^{-1} Mg_2Cl (Eurogentec), 1 U Red Diamond Taq DNA Polymerase (Eurogentec) and 0·2 mmol l^{-1} 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). 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-s 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 ver. 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 (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 no such strains were isolated from the negative control run without EC 682.

Statistical analysis

Significant changes as a response to the inclusion of DOX in the medium over a period of 6 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^{-1} DOX compared to the runs with the blank medium. Only population sizes starting from day 4 until day 9 were included for statistical analysis. *P*-values of significant results were calculated using ANOVA and the function lsmeans.

Results

Caecal bacterial dynamics at 0, 1 and 4 mg l^{-1} DOX

The EC 682 population size in the simulated porcine caecum was significantly higher ($P < 0{\cdot}001$) in reactors supplemented with 1 and 4 mg l^{-1} DOX (average increase of 1·20 ± 0·18 $\log_{10}$ CFU per ml and 1·19 ± 0·18 $\log_{10}$ CFU per ml respectively) compared to the blank controls (Fig. 1). However, no difference in population sizes of EC 682 was observed between the two DOX concentrations. In addition, a significant effect ($P < 0{\cdot}001$)

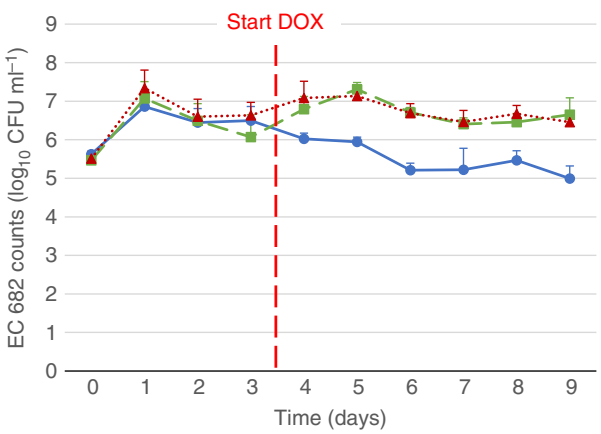

Figure 1 Enumeration of doxycycline (DOX)-resistant *Escherichia coli* strain EC 682 in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg l^{-1} DOX. Strain EC 682 (white colonies) was enumerated daily in duplicate on MacConkey no. 3 agar with 8 mg l^{-1} DOX (overnight incubation at 37°C) during 10 days. The population size of EC 682 is given in log transformed CFU per 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^{-1} DOX compared to blank medium. No significant difference in population size was observed between 1 and 4 mg l^{-1} DOX. [Colour figure can be viewed at wileyonlinelibrary.com]

of the factor 'time' on the population sizes of EC 682 was observed, since the EC 682 counts in blank medium decreased with time.

In the experiments with 1 mg l^{-1} DOX, the resistant coliforms (Fig. 2) showed a significantly ($P < 0{\cdot}001$) higher population size (average increase of $1{\cdot}17 \pm 0{\cdot}29$ $\log_{10}$ CFU per 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 (Fig. 3) were seen between any of the experiments, although the factor time did have a significant effect ($P = 0{\cdot}016$) on these population sizes.

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 (Fig. 4). A significantly higher population size of these anaerobes was found in the experiments with 4 mg l^{-1} compared to those with 1 mg l^{-1} DOX (average increase of $0{\cdot}42 \pm 0{\cdot}10$ $\log_{10}$ CFU per ml, $P = 0{\cdot}022$) and the blank controls (average increase of $0{\cdot}28 \pm 0{\cdot}10$ $\log_{10}$ CFU per ml, $P < 0{\cdot}001$).

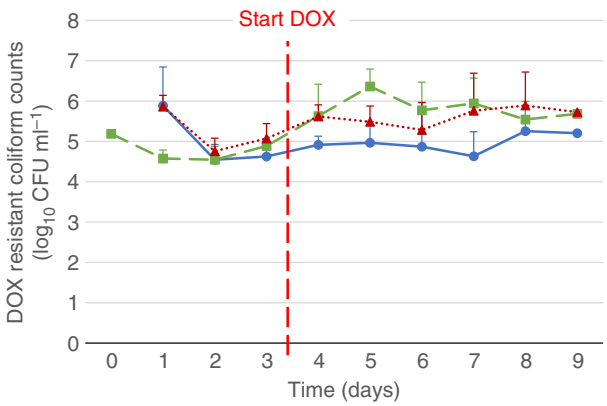

Figure 2 Enumeration of doxycycline (DOX)-resistant coliforms other than EC 682 in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg l^{-1} DOX. Resistant coliforms (red colonies) were enumerated daily in duplicate on MacConkey no. 3 agar with 8 mg l^{-1} DOX (overnight incubation at 37°C) during 10 days. The population size of resistant coliforms is given in log transformed CFU per 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^{-1} 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^{-1} DOX. The value given for day 0 of the experiments with 1 mg l^{-1} DOX, represents the count of only one of the three runs. The other two runs showed no growth at day 0. [Colour figure can be viewed at wileyonlinelibrary.com]

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^{-1} DOX ($n = 123$), those with 4 mg l^{-1} 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 2). These 420 *E. coli* isolates were assigned to 41 different clusters by rep-PCR. One cluster (no. 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).

Fifty-one of the 420 *E. coli* isolates carried the IncI1 replicon, of which four originated from the blank runs, 33 from the runs with 1 mg l^{-1} DOX and 14 from the runs with 4 mg l^{-1} DOX. All 51 isolates also carried the *tet*(A) gene (Table 2) and were assigned to 11 clusters (Table 3). None of these 51 isolates could be assigned to the large cluster (no. 8).

The 34 isolates that showed different morphology on RAPID' *E. coli* 2 agar than *E. coli*, were assigned to eight

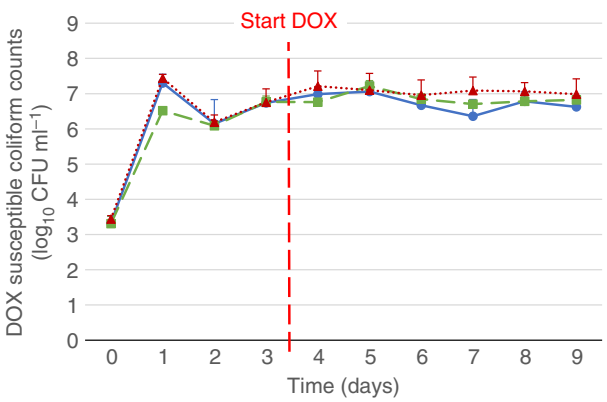

Figure 3 Enumeration of doxycycline (DOX) susceptible coliforms in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg l^{-1} DOX. Total coliforms and resistant coliforms (red colonies) were enumerated daily during 10 days in duplicate on MacConkey no. 3 agar without DOX and with 8 mg l^{-1} 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 per 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. [Colour figure can be viewed at wileyonlinelibrary.com]

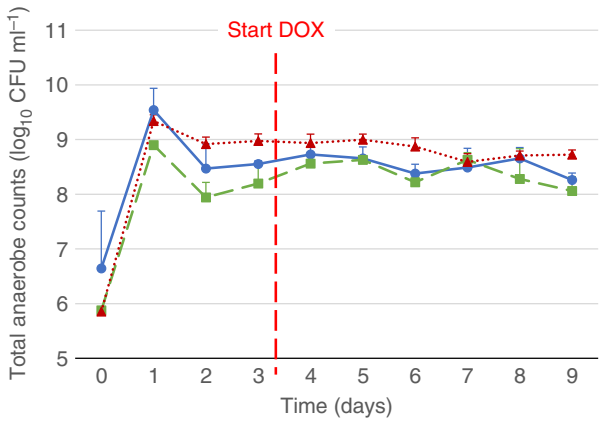

Figure 4 Enumeration of total anaerobes in the simulated pig caecum containing 0 (blank —●—), 1 (—■—) or 4 (···▲···) mg l^{-1} DOX. Total anaerobes were enumerated daily in duplicate on Reinforced Clostridial Agar without DOX (48 h anaerobic incubation at 37°C) during 10 days. The number of total anaerobes is given in log transformed CFU per 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^{-1} compared to those with 1 mg l^{-1} DOX and the blank controls. No other significant differences were seen. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Clustering of DOX resistant *Escherichia coli* by rep-PCR and *tet*(A)- and IncI1-carrying isolates

Run condition*	Number of isolates†	Clusters‡	*tet*(A)- and IncI1-carrying isolates (clusters)§
Blank	112	11	4 (2)
1 mg l^{-1} DOX	119	15	33 (5)
4 mg l^{-1} DOX	109	19	14 (4)

*For each condition, three runs were performed. Strains were isolated from all runs.
†*E. coli* strains isolated on day 3 (just before DOX administration), 5, 7 and 9.
‡Total number of detected Rep-PCR clusters.
§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).

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 three different clusters, did carry *tet*(A).

Table 3 Phenotypic resistance profile of *tet*(A)- and IncI1-carrying *Escherichia coli* isolates

Run condition	Cluster no. (a, b)*	Strain no.	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^{-1} 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^{-1} 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 no. 960 belonged to a cluster with eight *tet*(A) positive isolates and one *tet*(A) negative isolate, all originating from the negative control run.
‡Isolate no. 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^{-1} DOX.

Sixteen IncI1- and *tet*(A)-positive *E. coli* isolates (at least one isolate per cluster, see Table 3) were characterized with Sensititre. Eleven of these isolates showed the same resistance profile as EC 682 (Table 3). 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 3).

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 and Hughes 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^{-1} 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^{-1} was found. This finding could possibly be explained by the tetracycline resistance mechanism of EC 682, that is, 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^{-1} compared to 1 mg l^{-1} DOX, thus neutralizing the potential higher selective effect of 4 mg l^{-1} 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 specifically to determine the presence of transconjugants. The resistant coliform counts were also affected by DOX supplementation (Fig. 2), although statistical analysis only confirmed a selective effect in medium with 1 mg l^{-1} 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 and MacLean 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^{-1} DOX compared to 1 mg l^{-1} 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.e. 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 and MacLean 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 3). 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. Although, it should be noted 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^{-1} DOX with the same statistical significance were observed (Peeters *et al.* 2017). In addition, different studies confirm our finding that resistance genes conferring resistance to other antimicrobials than the one(s) administered can be coselected (Leverstein-van Hall *et al.* 2002; Looft *et al.* 2012; Agga *et al.* 2015). The selection of tetracycline genes on multidrug resistance plasmids obviously

contributes to a widespread dissemination of multidrug-resistant enteric bacteria.

In conclusion, caecal concentrations of DOX (1 and 4 mg l^{-1}) 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^{-1} DOX does not necessarily have a higher selective effect than 1 mg l^{-1} DOX, and previous observations indicate that very low antimicrobial concentrations (ng ml^{-1}) can select for persistent (*de novo*) resistance (Gullberg *et al.* 2011, 2014; Andersson and Hughes 2014), 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.

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Conflict of Interest

No conflict of interest declared.

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