



HAL
open science

Selection of antibiotic resistance in pigs after exposure to feed cross-contaminated with oxytetracycline

Cristina Santos-Santórum Suárez, Pascal Sanders, Murielle Gaugain, Alexis Viel, Frédéric Paboeuf, Jean-François Taillandier, Paméla Houée, Charlotte Valentin, Agnès Perrin-Guyomard

► To cite this version:

Cristina Santos-Santórum Suárez, Pascal Sanders, Murielle Gaugain, Alexis Viel, Frédéric Paboeuf, et al.. Selection of antibiotic resistance in pigs after exposure to feed cross-contaminated with oxytetracycline. *Veterinary Microbiology*, 2023, 287, pp.109924. 10.1016/j.vetmic.2023.109924. anses-04311332

HAL Id: anses-04311332

<https://anses.hal.science/anses-04311332>

Submitted on 28 Nov 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License



Selection of antibiotic resistance in pigs after exposure to feed cross-contaminated with oxytetracycline

Cristina Santos-Santórum Suárez^a, Pascal Sanders^a, Murielle Gaugain^a, Alexis Viel^a, Frédéric Paboeuf^b, Jean-François Taillandier^a, Paméla Houée^a, Charlotte Valentin^a, Agnès Perrin-Guyomard^{a,*}

^a ANSES-Fougères Laboratory (French National Agency for Food, Environment and Occupational Health & Safety), 10 B, rue Claude Bourgelat – Javené, CS 40608, 35306 CEDEX Fougères, France

^b ANSES-Ploufragan-Plouzané Laboratory (French National Agency for Food, Environment and Occupational Health & Safety), 22440 Ploufragan, France

ARTICLE INFO

Keywords:

Antibiotic
Antimicrobial resistance
Cross-contamination
Oxytetracycline

ABSTRACT

Due to possible cross-contamination of animal feedstuff with antibiotics, food-producing animals may be exposed to undesirable low concentrations of antimicrobials. These sub-therapeutic levels of antibiotics can lead to the selection of resistant bacteria in the animal gut. The goal of this study was to assess, through analysis of the faeces of treated and control pigs, the risk of resistant *E. coli* being selected after daily exposure for three weeks to feed contaminated with oxytetracycline at 1% of the therapeutic dose. Liquid Chromatography coupled to tandem Mass Spectrometry was used to determine the oxytetracycline concentrations in faecal samples. In the treated group, concentrations were in the range of 4481.9 – 8671.2 µg/kg. In the control group, these concentrations were either below the method's limit of quantification or up to 60.5 µg/kg. After a transient increase in resistance in both groups, microbiological analysis showed that the treated group had a significantly higher oxytetracycline resistance rate by the end of the study than the control group ($p < 0.001$). Furthermore, the treated animals were found to select co-resistances to nalidixic acid and ampicillin. Finally, at tolerated antibiotic contamination levels of feed, the treated group had a higher proportion of multidrug-resistant isolates at the end of the study than the control one ($p < 0.05$). The present study demonstrates that, at the tolerated contamination rates, both antimicrobial resistance and multidrug-resistant bacteria can be selected and evidenced in the gut microbiota.

1. Introduction

Antimicrobial resistance is currently a major issue that concerns both animal and human health. Even though the use of antibiotics as growth promoters in food-producing animals has been banned in the European Union since 2006 (European Commission, 2003), and despite the publication of guidelines for their proper use in veterinary medicine (World Health Organization, 2017), the potential presence of antimicrobials in animal feedstuff due to cross-contamination is a real concern. Indeed, because production lines are used both for medicated and non-medicated animal feedstuff, there may be a carry-over, leading to the unintended presence of trace antimicrobials in feed. This contamination, which appears unavoidable, can occur not only during feed fabrication, but also during transportation or storage either at the feed

mill or on the farm (Borràs et al. 2011; Stolker et al. 2013; Filipitzi et al. 2016). This problem has already been reported in several studies that have demonstrated the undesirable presence of low concentrations of antibiotics in animal feedstuff (Lynas et al. 1998; Robert et al. 2016; Gaugain et al. 2020; Przeniosło-Siwczyńska et al. 2020).

In France, cross-contamination of feed with antibiotics is regulated by the Good Manufacturing Practices published by the Ministry of Agriculture and Fisheries. This document establishes a maximum contamination rate of 5% for the first batch following production of medicated feed, and 1% for the next one (French Ministry of Agriculture and Fisheries. 2007).

Between 2017 and 2018, monitoring plans were carried out in France during which 100 pig, poultry and rabbit feed samples were collected each year from different farms and analysed for 11

* Corresponding author.

E-mail address: agnes.perrin-guyomard@anses.fr (A. Perrin-Guyomard).

<https://doi.org/10.1016/j.vetmic.2023.109924>

Received 29 June 2023; Received in revised form 18 October 2023; Accepted 20 November 2023

Available online 22 November 2023

0378-1135/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

antimicrobials. The most frequently found antibiotics were oxytetracycline, sulfadiazine and sulphadimethoxine (Gaugain et al. 2020).

The exposure of animals to contaminated feed may lead to the presence of antimicrobial residues in food of animal origin, even possibly to levels above the maximum residue levels (MRLs) for some antibiotics. Furthermore, the consumption of low antibiotic concentrations by animals can play an important role in the selection of resistant bacteria in the gut microbiota.

It is well known that therapeutic concentrations of antibiotics can select for resistant bacteria. However, literature on how sub-therapeutic levels of antimicrobials can also select for resistant mutants is still scarce (EFSA. 2021).

Some studies have demonstrated in vitro that levels of antibiotics under the minimum inhibitory concentration (MIC) can select *Escherichia coli*, *Salmonella enterica* or *Pseudomonas aeruginosa* (Gullberg et al. 2011; Jørgensen et al. 2013; Gullberg et al. 2014; Peeters et al. 2018; Moore et al. 2021), or can lead in vivo to specific selection in the gastrointestinal bacterial populations of pigs (Holman and Chénier 2013; Lin et al. 2017).

Recently, the European Food Safety Authority (EFSA) developed different approaches to predict the antimicrobial levels in non-medicated feed that would not result in the emergence and/or selection of resistance. However, these approaches are based on the very limited data available on the specific link between a low concentration of antimicrobial in feed and the selection and development of resistance to antibiotics relevant for human and animal health in the gut microbiota of animals (EFSA. 2021). Therefore, the goal of this study was to produce new in vivo data in order to evaluate the risk of selection of antimicrobial resistance in pig intestinal microbiota after exposure to feed contaminated with low concentrations of oxytetracycline.

2. Materials and methods

2.1. Animal study design and sample collection

An animal study, approved by the French Committee on Animal Research and Ethics, was carried out with 12 pigs. Specific-pathogen-free (SPF) pigs were provided by ANSES's Ploufragan laboratory (France). The pigs were randomly divided into two groups of six animals each: a control group and a treated one. To avoid any design bias, both groups were housed in the same room, and all the animals belonging to the same group were kept together in the same box. For 18 days, the treated group received feed contaminated with an oxytetracycline (OTC) premix (OTC 40-CR from Huvepharma, Segré-en-Anjou Bleu, France) at a concentration corresponding to 1% of the recommended therapeutic dose (i.e. 0.25 g premix/kg feed/day). This dose is tolerated by the Good Manufacturing Practices for animal feed production (French Ministry of Agriculture and Fisheries. 2007). Hence, the administered dose was 10 mg of OTC/kg of feed per day (i.e. 0.5 mg/kg body weight/day). Feed intake was monitored and corresponded to 50 g per kg of body weight per day and the daily dose was divided into two administrations. Finally, in order to adjust the administered dose of premix and the amount of feed required, the animals were weighed every week and the mean of these values was used to adapt the quantities accordingly.

Faeces from each animal were sampled after spontaneous defecation four days before the treatment (T0), and four times during the 3-week OTC treatment (on days 3, 7, 10 and 17 after the first day of OTC administration, i.e. T1, T2, T3 and T4). The fresh faecal samples were processed as follows: 5 ± 0.5 g was weighed and diluted to 1:10 by adding 45 ml of 0.9% saline solution (Labocea, Fougères, France) containing 10% of glycerol (Dutscher, Bernolsheim, France). These samples were homogenised with a Stomacher® and the filtered suspensions were stored at – 80 °C before microbiological analysis. The remaining faecal samples were stored at – 80 °C before the extraction and OTC assay.

2.2. Microbiological analysis

2.2.1. Enumeration of total and resistant *E. coli* populations and assessment of the resistance rate

The fresh faecal samples were serially diluted in saline solution to obtain 10⁻² and 10⁻⁴ dilutions. Fifty µl of both dilutions in addition to the original (undiluted) samples was inoculated in duplicate with the Eddy Jet spiral automatic plater (IUL S.A., Barcelona, Spain) onto MacConkey agar (Labocea, Fougères, France) plates that contained either no antibiotic or OTC at 8 µg/ml. This OTC concentration corresponds to the epidemiological cut-off value (ECOFF) established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) that allows a distinction to be made between a wild-type (WT) and non-wild type (NWT) ('microbiological' resistant) microorganism (<http://www.eucast.org>, last accessed 09/11/2022). Samples were then incubated at 44 °C for 18–24 h. After incubation, presumptive *E. coli* colonies were counted with the SphereFlash® automatic colony counter (IUL S. A., Barcelona, Spain) to estimate the total population and the NWT population. The OTC resistance rate, being the NWT/Total ratio between the mean of NWT *E. coli* colonies from plates containing OTC and the mean of total *E. coli* colonies from plates with no antibiotic was calculated for each pig. The mean of all NWT/Total ratios was then calculated in order to assess the OTC resistance rate in each group at each time point.

2.2.2. MIC determination and antimicrobial susceptibility testing

Six colonies of presumptive *E. coli* were randomly selected and sub-cultured from each antibiotic-free MacConkey agar plate on Brilliance™ agar plates (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) for purification. Isolates were stored at – 80 °C until further analysis. The minimum inhibitory concentration of amikacin (AMK), ampicillin (AMP), azithromycin (AZM), cefotaxime (CTX), ceftazidime (CAZ), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), gentamicin (GEN), meropenem (MEM), nalidixic acid (NAL), sulfamethoxazole (SMX), tetracycline (TET), tigecycline (TIG) and trimethoprim (TMP) was assessed for each isolate by broth micro-dilution, using EUVSEC3 Sensititre™ EU Surveillance Plates (ThermoFisher Scientific, Illkirch-Graffenstaden, France). Antimicrobial susceptibility was assessed using the ECOFF value and the colonies were classified as WT/NWT. Multidrug-resistant (MDR) isolates, i.e. isolates resistant to at least three of the antimicrobial classes described above, were enumerated. Resistance to both CTX and CAZ and to both CIP and NAL are, respectively, addressed together.

2.2.3. Determination of the phylogenetic groups

A heat shock method was used to extract DNA from the six isolates from each animal at each sampling time. The phylogenetic group was then assigned by PCR according to Clermont et al. (2013) (Fig. 1).

2.3. Faecal extraction and LC-MS/MS analysis

In order to quantify the concentrations of OTC in faecal samples, a solid phase extraction (SPE) followed by LC-MS/MS analysis was performed as previously described (Santos-Santórum Suárez et al. 2022). The method's lower limit of quantification (LLOQ) was 20 µg/kg.

2.4. Statistical tests

A two-way analysis of variance (ANOVA) was used to determine whether the treatment and the time had a significant impact on the OTC resistance rate among the *E. coli* population. The interaction between treatment and time was also assessed. Logistic regression models were then developed based on the antimicrobial susceptibility of the isolates (MICs) and their classification into WT or NWT. The goal of these models was to evaluate the potential effect of treatment and time on the selection of co-resistances, as well as to analyse MDR profiles. Like for the

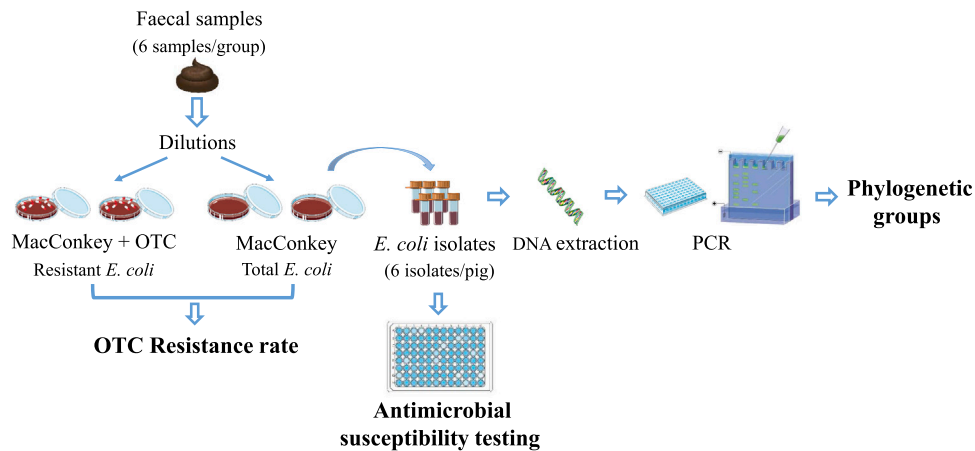


Fig. 1. Schematic representation of the complete microbiological analysis. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported licence.

ANOVA, the interaction was tested. Fisher's exact test was also performed in order to compare the proportion of resistant isolates between both groups at T0 and T4.

A repeated measures ANOVA (RM-ANOVA) was performed so as to compare OTC concentrations in faeces between the treated animals and over time.

All the tests were performed with GraphPad Prism software (version 9.4.0) and R software (version 4.1.2). A p-value of 0.05 was taken as the limit of significance.

3. Results

3.1. Microbiological analysis

3.1.1. Total *E. coli* faecal population

Before the beginning of the treatment (T0), the mean total *E. coli* faecal population was of 6.7 log₁₀ CFU/g in the treated group and 7.02 log₁₀ CFU/g in the control one. They were not significantly different ($p > 0.05$). Nine days after arrival (between T0 and T1), all the pigs, but especially those belonging to the control group, had diarrhoea symptoms for four days. The presence of *Rotavirus* was verified but the test was negative for all the animals. At T1, the *E. coli* population in the control group significantly increased to 8.93 log₁₀ CFU/g ($p < 0.05$ when compared with T0), while it remained stable in the treated group (7.56 log₁₀ CFU/g, $p > 0.05$ when compared with T0), leading to significantly different concentrations between control and treated animals ($p < 0.05$). From T2 onwards (after the diarrhoea episode), the faecal *E. coli* population returned to its initial level in the control animals and remained stable in both groups until the end of the experiment, with no significant differences between the two groups ($p > 0.05$).

3.1.2. Oxytetracycline resistance rate in the *E. coli* faecal population

Before the beginning of the treatment, both treated and control groups had a low OTC resistance rate in the faecal *E. coli* population (a mean ratio of 0.078 ± 0.03 in the treated group, and of 0.248 ± 0.32 in the control group, see Fig. 3) but with great variability between pigs. As for the total *E. coli* population, this resistance rate briefly increased in the control group, reaching 0.818 at T1 without significance when compared with T0 ($p > 0.05$). A huge drop was then observed between T1 and T2 to reach a low resistance rate (0.078) which then stayed generally stable until the end of the experimental study. On the other hand, in the treated group, resistance rates constantly increased over the whole period, reaching a significant difference at T4 compared with T0 ($p < 0.01$). At the end of the study, the difference in the OTC resistance rate in the two groups was also highly significant ($p < 0.001$), the rate being 0.893 in the treated group and 0.093 in the control group. The

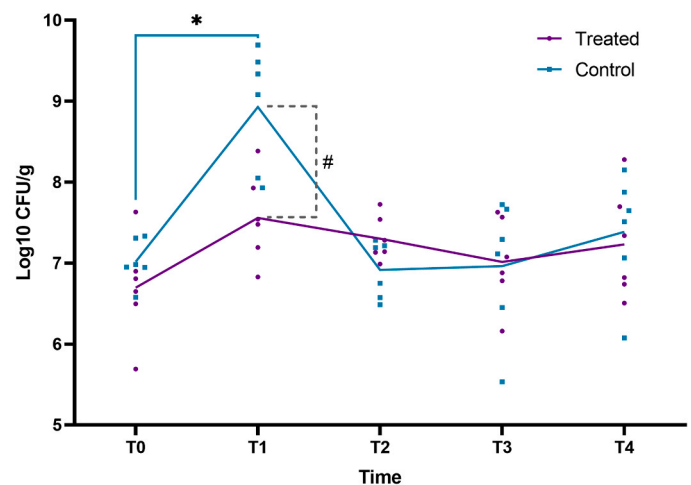


Fig. 2. Total faecal *E. coli* population count. T0 = day 4 before the beginning of the treatment; T1 = day 3 of treatment; T2 = day 7 of treatment; T3 = day 10 of treatment; T4 = day 17 of treatment. *: $p < 0.05$ between T0 and T1 in the control group. #: $p < 0.05$ between the treated and the control group at T1.

interaction between time and treatment was also significant ($p < 0.001$).

3.1.3. Antimicrobial susceptibility testing and co-resistance assessment

A total of 360 colonies were assessed for MIC testing (36 in each group at each time point). As shown in Fig. 4, a maximum of 11% of the colonies had an MIC to TET above the ECOFF in the control group taking the whole study into account, except for the transient event at T1, where 100% of the colonies were resistant to tetracycline. In the treated group, the number of *E. coli* isolates with an MIC to TET above the ECOFF value (8 mg/L) increased to reach 83% of resistance at T4 (with the vast majority of isolates having a high MIC $> 32 \mu\text{g/ml}$), highlighting the treatment's significant impact in selecting resistance to TET taking the whole study period into account ($p < 0.001$). The interaction between the group status and time was also significant ($p < 0.001$).

In the case of NAL, although OTC treatment seemed to play a role in the selection of NAL resistance taking the whole study into account, no significant difference was observed at T4 between both groups ($p > 0.05$). In the control group, some of the isolated strains are less susceptible but not resistant at T1.

In the control group, the number of *E. coli* resistant to AMP and TMP increased at T1 and decreased sharply at T2 and then regularly. The two distributions are not significantly different ($p > 0.05$) over the time

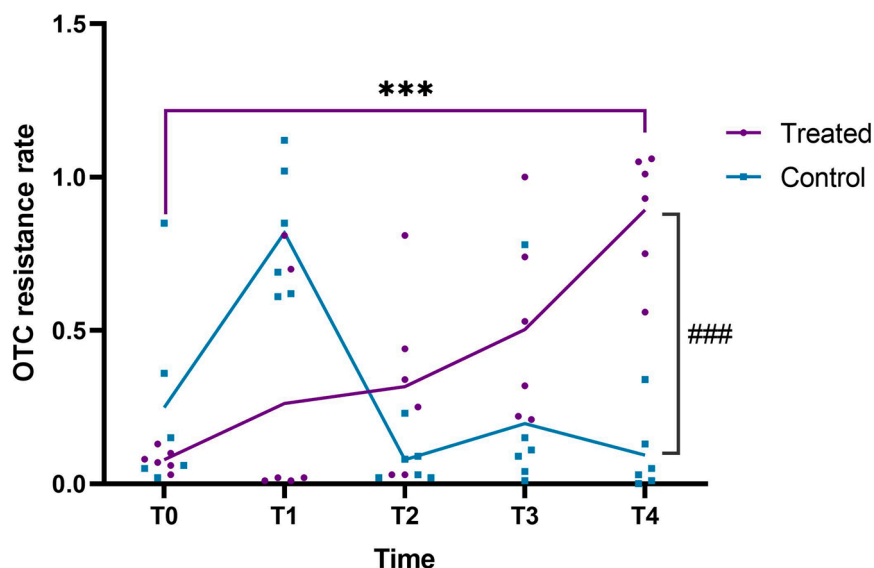


Fig. 3. Evolution of the average antimicrobial resistance rate to OTC in the *E. coli* faecal population over time in the treated and control groups. T0 = day 4 before the beginning of the treatment; T1 = day 3 of treatment; T2 = day 7 of treatment; T3 = day 10 of treatment; T4 = day 17 of treatment. ***: $p < 0.001$ between T0 and T1 in the treated group. ###: $p < 0.001$ between the treated and the control group at T4.

exposure ($p > 0.05$). Nevertheless, a significant difference between both groups was observed at T4 for AMP ($p < 0.05$) while for TMP, this difference was very close to the limit of significance ($p = 0.055$). These differences were not present at the beginning of the study ($p > 0.05$).

Conversely, the number of *E. coli* isolates resistant to chloramphenicol was significantly higher ($p < 0.001$) in the control group than in the treated group when considering the whole study period. However, there was no significant difference at the end of the study between the two groups ($p > 0.05$).

No significant differences between the control and treated groups were observed for CIP and SMX taking the whole period of study into account ($p > 0.05$, data not shown).

Only one strain isolated from a treated animal was resistant to CTX (at T1) and only one from a control animal had an MIC to GEN above the ECOFF (at T4). No isolated *E. coli* resistant to AMK, AZM, CAZ, COL, MEM and TIG were observed during the treatment period in either of the two groups (data not shown).

All MDR results are shown in Fig. 5.

In the control group, only three of the isolated colonies were MDR before the beginning of the study (T0), all of them having a resistance profile corresponding to three antimicrobial families. On day 3, when the animals had diarrhoea, all the colonies were resistant to six different antibiotics with the same MDR profile, i.e. AMP-CHL-(fluoro)quinolone-SMX-TET-TMP resistances. On day 7, the number of MDR isolates decreased to seven and continued to decrease to five on day 10 and two at the end of the study. Between day 7 and day 17, all MDR isolates were resistant to three antimicrobial families.

In the treated group, five of the *E. coli* isolates were MDR, before administration of OTC (T0), of which three were resistant to three antimicrobials and two were resistant to four families of antibiotics. On day 3 after the beginning of OTC administration (T1), the number of MDR colonies increased to 14, and more than half of them (11 isolates) were resistant to more than three different antimicrobials, the majority of which (8 of the 36 isolated *E. coli* colonies) had a resistance profile to six antibiotics. The number of MDR strains isolated on day 7 (T2) increased slightly (15), but isolated strains were mostly resistant to only three antibiotics. On day 10 (T3), the number of MDR *E. coli* continued to increase (20), with 14 strains resistant to three antibiotics, five resistant to four antibiotics and one resistant to five antibiotics. Finally, at the end of the study (day 17, T4), six of the colonies were resistant to three antimicrobial families and five were resistant to four classes of

antibiotics. The most frequently observed MDR profiles were AMP-(fluoro)quinolone-TET at T0, AMP-CHL-(fluoro)quinolone-SMX-TET-TMP at T1, AMP-(fluoro)quinolone-TET at T2 and T3 and AMP-SMX-TET-TMP at T4.

OTC administration had no significant impact in the selection of MDR profiles ($p > 0.05$) taking the whole study period into account. However, there was a significant difference in the proportion of MDR *E. coli* between both groups by the end of the study ($p < 0.05$, T4), in contrast to the beginning (T0) ($p > 0.05$).

3.1.4. Phylogenetic group determination

All the phylotyping results are shown in Fig. 6. At the beginning of the study (T0), *E. coli* isolates belonging to phylogroups A and B1 were dominant in the control group (19 and 11, respectively) and in the treated group (18 and 14, respectively). Phylogroups B2 and E were also identified in lower proportions among *E. coli* isolated from pigs in the control group (5 and 1, respectively) as were phylogroups B2, C and D from pigs in the treated group (1, 2 and 1, respectively).

In the control animals, all the isolates belonged to group A at T1. This number then decreased between T2 and T4 to reach 18 colonies, the rest of the isolates belonging to phylogroup E (eight) phylogroup B1 (five) and phylogroup B2 (four). One isolate (out of 360) could not be assigned to any of the existing phylogroups.

In the treated animal group, the amount of phylogroup A isolates decreased from 17 to eight from T1 to T3, while phylogroup B1 isolates increased from 15 to 22. At the end of the study, phylogroup A returned to a level similar to that at the start of the experiment (20 isolates) in the treated group, followed by phylogroup B1 (eight isolates) and phylogroup C (eight isolates).

As shown in Fig. 7, all the MDR isolates belonged to phylogroups A, B1 or C. In isolates belonging to phylogroup A, the most common MDR profile was AMP-CHL-(fluoro)quinolone-SMX-TET-TMP resistances for both control and treated groups. For phylogroup B1, the most abundant MDR profile was AMP-(fluoro)quinolone-TET for the treated animals, while for the control ones the dominant profiles were AMP-CHL-TET and AMP-SMX-TMP, even though AMP-(fluoro)quinolone-TET resistances were also present. Finally, for phylogroup C the only observed MDR profile was AMP-SMX-TET-TMP, identified exclusively in the treated group.

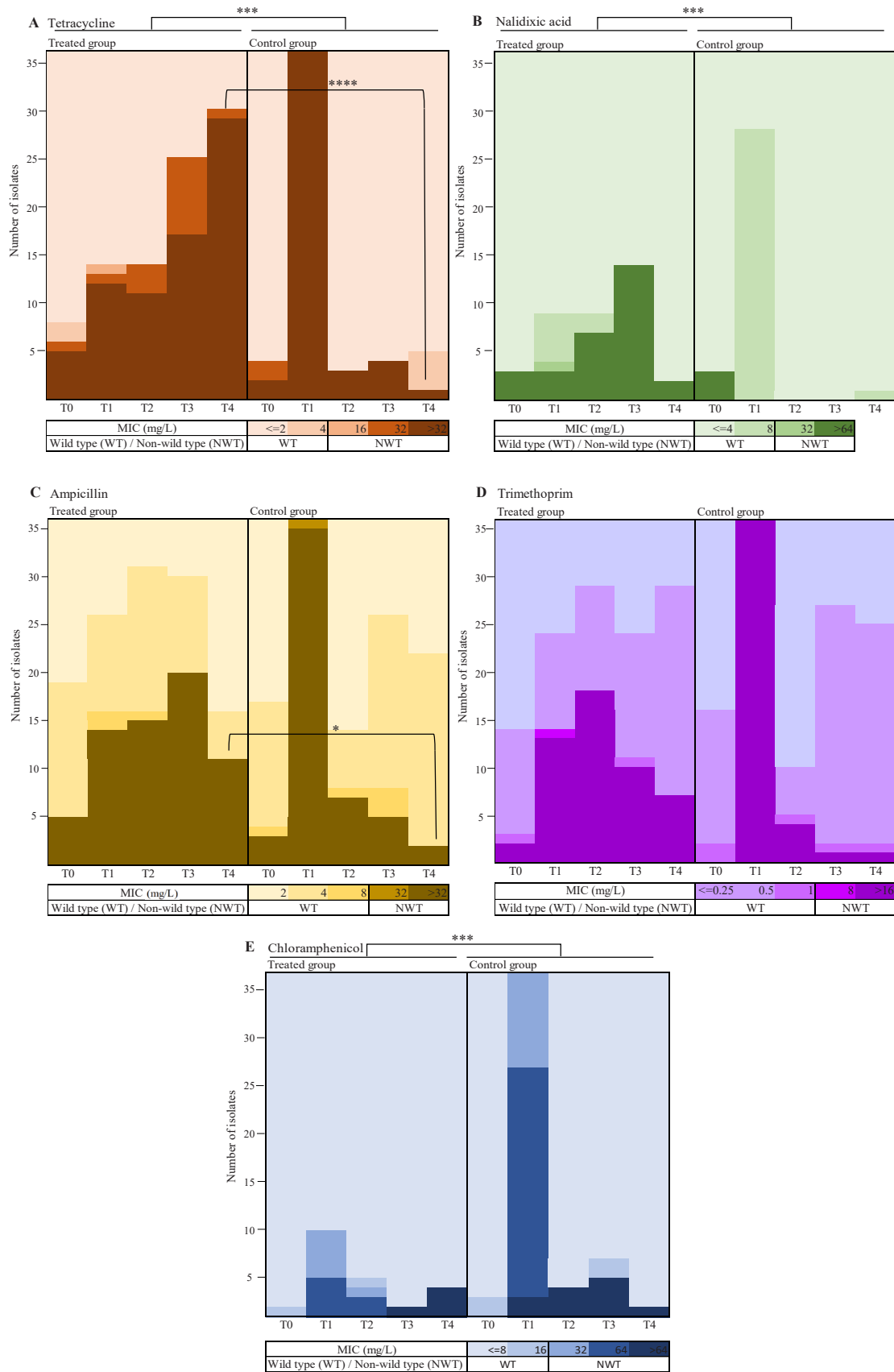


Fig. 4. Distribution of the minimum inhibitory concentrations (MICs) of tetracycline (A), nalidixic acid (B), ampicillin (C), trimethoprim (D) and chloramphenicol (E) in the treated and control groups over time. * = $p < 0.05$ *** = $p < 0.001$ **** = $p < 0.0001$.

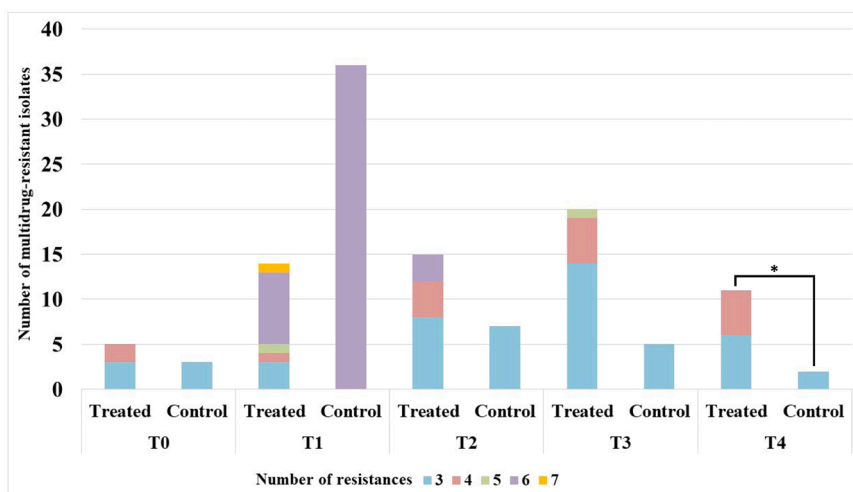


Fig. 5. Multidrug resistance profile of the *E. coli* isolates. * =p < 0.05.

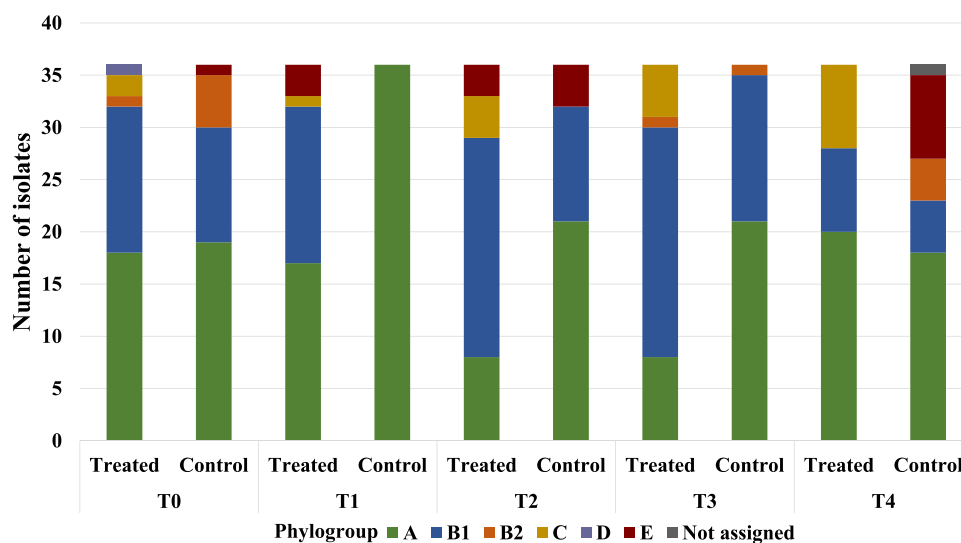


Fig. 6. Distribution of *E. coli* isolates in the phylogroups.

3.2. Oxytetracycline extraction and quantification by LC-MS/MS

The concentrations of OTC in the faeces of animals at each sampling timepoint are shown in Table 1. Before the beginning of the treatment (T0), trace amounts of OTC were observed in the faecal samples of the treated group. Then, at T1 the faecal concentration of OTC increased and remained between 4481.9 and 8671.2 µg/kg until the end of the study. No significant difference was observed between the animals belonging to the treated group over time (p > 0.05). In the control group, the concentrations of OTC were below the LLOQ (20 µg/kg) before the beginning of the treatment (T0). Between T1 and T3, trace amounts of OTC were detected in the faecal samples from some of the control animals (less than 60 µg/kg), which then decreased and remained below the LLOQ again at T4.

4. Discussion

Cross-contamination by antibiotics could occur between medicated animal feedstuff to non-target feed. Maximum levels of cross-contamination are regulated by the Good Manufacturing Practices. The goal of this study was to evaluate the impact of these levels in the selection of antibiotic resistance in pigs exposed to a contaminated feed.

The microbiological analysis performed on the faecal samples showed that, taking the whole study into account, there was no difference in the size of the total *E. coli* population between both treated and control groups over time, except for a transient episode of diarrhoea experienced by the animals in the control group and some of the treated ones. The illness, which could have been caused by the change in the animals' environment or by their postweaning status (age), led to a perturbation in their microbiota, increasing both total and resistant *E. coli* populations in the control group. This kind of dysbiosis with an increase in *Enterobacteriaceae* associated with postweaning diarrhoea has already been reported previously (Dou et al. 2017). Furthermore, diarrhoea symptoms induced by pathogenic *E. coli* have already been reported in weaned pigs (Fairbrother et al. 2005; Tran et al. 2018). As this event affected both groups and all the animals were housed in the same room and thus living in the same conditions, a statistical comparison of the results is possible.

The uninterrupted exposure of pigs to feed contaminated with OTC, hence to low antimicrobial concentrations in feed, resulted in a highly significant increase in the resistance rate to OTC (and thus to TET) of *E. coli* in the gut microbiota of treated animals. To our knowledge, no in vivo studies have previously demonstrated the selection of resistant *E. coli* after exposure to a tetracycline at subtherapeutic levels. Our

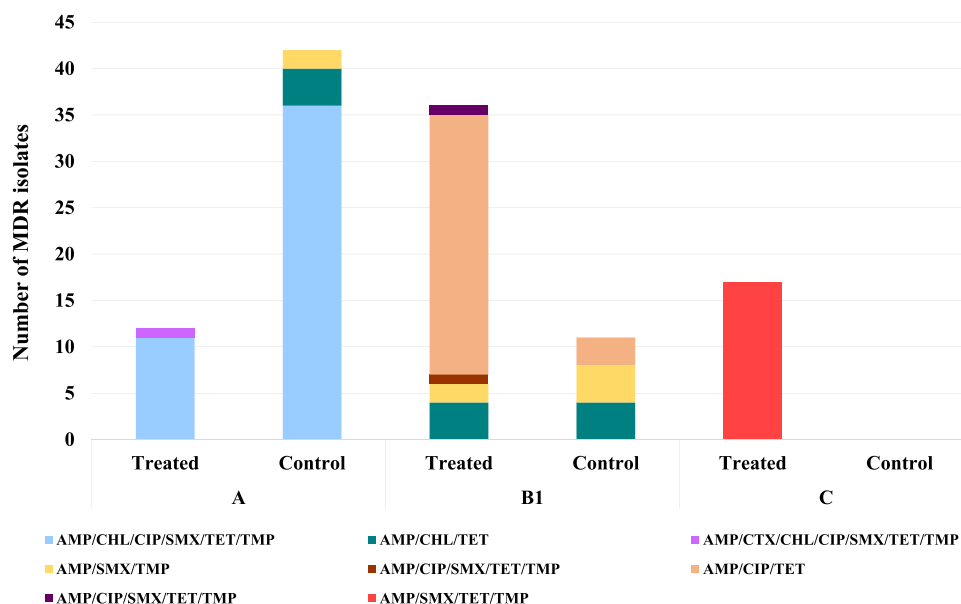


Fig. 7. Correlation between phylogenetic groups and the profile of multidrug-resistant isolates.

Table 1

Mean concentration and standard deviation of oxytetracycline in faeces (in $\mu\text{g}/\text{kg}$) in the treated and control groups. LLOQ=lower limit of quantification.

Day	Treated group Mean ($\mu\text{g}/\text{kg}$) \pm standard deviation	Control group Mean ($\mu\text{g}/\text{kg}$) \pm standard deviation
T0	27.78 \pm 11.25 ^a	<LLOQ
T1	6491.95 \pm 1430.6	24.12 \pm 9.97 ^a
T2	7257.74 \pm 1524.3	46.19 \pm 12.11
T3	6673.83 \pm 317.6	23.36 \pm 6.64 ^a
T4	6064.80 \pm 1381.5	<LLOQ

^a Uncertainty of the results unknown because some of the samples are below the LLOQ

results agree with what Holman and Chénier (Holman and Chénier 2013) reported in their study: after exposure of pigs to subtherapeutic levels of tylosin, a significant increase in tylosin-resistant anaerobes was observed compared with the control group. Similarly, Lin et al. (Lin et al. 2017) conducted an in vivo study in which they compared the effect of two antimicrobials (ceftiofur and enrofloxacin) at therapeutic and sub-therapeutic levels on *E. coli* gastrointestinal sub-populations in pigs. They observed that the groups treated with sub-therapeutic concentrations of antibiotics showed significantly higher rates of resistant *E. coli* than the therapeutic or control groups.

The LC-MS/MS analysis of faecal samples revealed relatively high concentrations (around 6000–7000 $\mu\text{g}/\text{kg}$) of OTC in the treated group compared with the control group. This agrees with what is described in the literature about OTC having low oral bioavailability and absorption (Mevius et al., 1986; Nielsen and Gyrd-Hansen, 1996). Using an ECOFF of 8000 $\mu\text{g}/\text{L}$, we could imagine that the gut's bacterial population could be exposed to OTC concentrations below this epidemiological limit, especially if there is binding to the intestinal content, which occurs for tetracycline in humans (Ahn et al. 2018), and that this could increase the risk of selection of resistance. Moreover, in vitro competition approaches between isogenic susceptible and resistant bacteria have demonstrated that exposure to a range of antibiotic level between the Minimum Selective Concentration (MSC) and the MIC of the sensitive strain represents a sub-MIC selective window in which resistant bacteria have an advantage. Sensitive bacteria are still able to grow and thus a wider spectrum of resistant mutants can emerge. This means that sub-MIC doses of antibiotics can select for more stable mutants than high

doses, i.e. these mutations are less likely to be reversed after exposure to the antibiotic. These mutations are likely to have a low fitness cost and would therefore be more stable, even in the absence of the antibiotic (Gullberg et al. 2011; Andersson and Hughes, 2014; Sandegren, 2014). Furthermore, these antibiotic residues could spread through the environment, representing a risk for public health. In the control group, traces of OTC were detected between days 3 and 10, probably due to contamination in the boxes where the animals were located. However, these concentrations are negligible when compared with those found in the treated group (more than 150 times lower) and they did not lead to an increase in the resistance rate.

In our conditions, due to the presence of very low levels of residues in the control group, we could also conclude that a low concentration around 20 $\mu\text{g}/\text{kg}$ in faeces does not lead to resistance selection.

Selection of resistance to the antimicrobial that was used is expected, but co-selection of resistance to other antibiotics can also occur when other AMR genes are located on the same genetic element than genes involved in OTC resistance. Interestingly, an increase in the resistance rate to antimicrobial drugs from other families, such as nalidixic acid and ampicillin, was also observed in the treated group, with a tendency also observed for trimethoprim. New studies should investigate this further. Similar results were observed by do V. Barroso et al. (do V. Barroso et al. 2022) in whose study co-resistance to doxycycline, cephalexin and kanamycin was selected after pigs' exposure to sub-therapeutic levels of a combination of colistin and tylosin in feed.

In the present study, selection of co-resistances to other antimicrobial families led to selection of MDR *E. coli* in the treated group after three weeks of exposure to low concentrations of OTC when comparing to the control group. The results show that the exposure of animals to sub-MIC concentrations of an antibiotic via contaminated feed may not only select *E. coli* resistant to the administered antibiotic, but also to other families of antimicrobials such as sulphonamides, penicillins, (fluoro)quinolones and diamynopyrimidines. These resistant bacteria may then be disseminated to humans or into the environment.

Phylogenetic analysis showed that the most frequently identified groups were A and B1. Furthermore, all the resistant or MDR strains belonged to groups A, B1 or C, which suggests that these resistant *E. coli* isolates in the animal gut were commensal (Clermont et al. 2013). Moreover, in addition to having the same MDR profile, all the strains from the control group at T1 belonged to phylogroup A. This may mean that the diarrhoea symptoms led to dominance of a potential *E. coli* clone

in the gut microbiota or, on the contrary, this potential clone could have induced the disease (Denamur et al. 2021). Further sequencing studies should be performed to check whether these strains are clones or not.

5. Conclusion

The present study showed that the exposure of food-producing animals to feed cross-contaminated with OTC lead to the selection of OTC-resistant *E. coli* in the gut microbiota. Moreover, co-resistance may also be selected, leading to multidrug- resistance profiles. In addition, antibiotic residues can be disseminated in the environment through animal faeces. In conclusion, the results of this study showed that cross-contamination of animal feedstuff with antibiotics can aggravate the antimicrobial resistance problem, which is currently a major public health issue.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Michel Laurentie, Jérôme Blot, Jérôme Henri and Antoine Huguet for their help and support.

References

- Ahn, Y., Jung, J.Y., Veach, B.T., Khare, S., Gokulan, K., Piñeiro, S.A., Cerniglia, C.E., 2018. In vitro test systems to determine tetracycline residue binding to human feces. *Regul. Toxicol. Pharm.* 99, 105–115.
- Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12 (7), 465–478.
- Borràs, S., Companyó, R., Granados, M., Guiteras, J., Pérez-Vendrell, A.M., Brufau, J., Medina, M., Bosch, J., 2011. Analysis of antimicrobial agents in animal feed. *Trends Anal. Chem.* 30 (7), 1042–1064.
- Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* 5 (1), 58–65.
- Denamur, E., Clermont, O., Bonacorsi, S., Gordon, D., 2021. The population genetics of pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 19 (1), 37–54.
- do V. Barroso, M., da Silva, J.S., Moreira, S.M., Sabino, Y.N.V., Rocha, G.C., Moreira, M. A.S., Bazzolli, D.M.S., Mantovani, H.C., 2022. Selection of Multidrug-Resistant Enterobacteria in Weaned Pigs and Its Association With In-feed Subtherapeutic Combination of Colistin and Tylosin. *Curr. Microbiol.* 79 (11), 349.
- Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakkhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A., Guyonvarch, A., et al., 2017. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS One* 12 (1), e0169851.
- EFSA, 2021. Maximum levels of cross-contamination for 24 antimicrobial active substances in non-target feed. Part 1: Methodol., Gen. data gaps uncertainties 1831–4732.
- European Commission. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, 2003.
- Fairbrother, J.M., Nadeau, E., Gyles, C.L., 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim. Health Res. Rev.* 6 (1), 17–39.
- Filippitzi, M.E., Sarrazin, S., Imberechts, H., Smet, A., 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* 33 (4), 644–655.
- French Ministry of Agriculture and Fisheries, 2007. Décision du 12 février 2007 relative aux bonnes pratiques de fabrication et de distribution en gros des aliments médicamenteux. *J. Off. De. la République Fr.*
- Gaugain, M., Fourmond, M.P., Fuselier, R., Verdon, E., Roudaut, B., Pessel, D., 2020. Control of Antimicrobials in Feed Using Liquid Chromatography-Tandem Mass Spectrometry: Assessment of Cross-Contamination Rates at the Farm Level. *J. Agric. Food Chem.* 68 (34), 9033–9042.
- Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio* 5 (5) e01918-01914.
- Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7 (7), e1002158.
- Holman, D.B., Chénier, M.R., 2013. Impact of subtherapeutic administration of tylosin and chlortetracycline on antimicrobial resistance in farrow-to-finish swine. *FEMS Microbiol. Ecol.* 85 (1), 1–13.
- Jørgensen, K.M., Wassermann, T., Jensen, P., Hengzuang, W., Molin, S., Høiby, N., Ciofu, O., 2013. Sublethal ciprofloxacin treatment leads to rapid development of high-level ciprofloxacin resistance during long-term experimental evolution of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 57 (9), 4215–4221.
- Lin, D., Chen, K., Xie, M., Ye, L., Chan, E.W.-C., Chen, S., 2017. Effect of ceftiofur and enrofloxacin on *E. coli* sub-population in pig gastrointestinal tract. *J. Glob. Antimicrob. Resist.* 10, 126–130.
- Lynas, L., Currie, D., McCaughey, W.J., McEvoy, J.D.G., Kennedy, D.G., 1998. Contamination of animal feedingstuffs with undeclared antimicrobial additives. *Food Addit. Contam.* 15 (2), 162–170.
- Mevius, D.J., Vellenga, L., Breukink, H.J., Nouws, J.F., Vree, T.B., Driessens, F., 1986. Pharmacokinetics and renal clearance of oxytetracycline in piglets following intravenous and oral administration. *Vet. Q* 8 (4), 274–284.
- Moore, J.E., Millar, B.C., Ollman-Selinger, M., Cambridge, L., 2021. The role of suboptimal concentrations of nebulized tobramycin in driving antimicrobial resistance in *Pseudomonas aeruginosa* isolates in cystic fibrosis. *Respir. Care* 66 (9), 1446–1457.
- Nielsen, P., Gyrd-Hansen, N., 1996. Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs. *J. Vet. Pharm. Ther.* 19, 305–311.
- Peeters, L.E.J., Croubels, S., Rasschaert, G., Imberechts, H., Daeseleire, E., Dewulf, J., Heyndrickx, M., Butaye, P., Haesebrouck, F., Smet, A., 2018. Effect of residual doxycycline concentrations on resistance selection and transfer in porcine commensal *Escherichia coli*. *Int J. Antimicrob. Agents* 51 (1), 123–127.
- Przeniosło-Siwczyńska, M., Patyra, E., Grelik, A., Chylik-Purchala, M., Kozak, B., Kwiatek, K., 2020. Contamination of animal feed with undeclared tetracyclines—confirmatory analysis by liquid chromatography-mass spectrometry after microbiological plate test. *Mol. (Basel, Switz.)* 25 (9).
- Robert, C., Brasseur, P.-Y., Dubois, M., Delahaut, P., Gillard, N., 2016. Development and validation of rapid multiresidue and multi-class analysis for antibiotics and anthelmintics in feed by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry. *Food Addit. Contam.: Part A* 33 (8), 1312–1323.
- Sandegren, L., 2014. Selection of antibiotic resistance at very low antibiotic concentrations. *Ups. J. Med Sci.* 119 (2), 103–107.
- Santos-Santórum Suárez, C., Sanders, P., Perrin-Guyomard, A., Hurtaud-Pessel, D., Laurentie, M., Viel, A., Taillandier, J.-F., Lagree, M.-P., Gaugain, M., 2022. Validation of a LC-MS/MS method for the quantitative analysis of four antibiotics in pig tissues and plasma to assess the risk of transfer of residues to edible matrices after exposure to cross-contaminated feed. *Food Addit. Contam.: Part A* 39 (11), 1818–1827.
- Stolker, A.A., Manti, V., Zuidema, T., van Egmond, H., Deckers, E.R., Herbes, R., Hooglugt, J., Olde Heuvel, E., de Jong, J., 2013. Carry-over of veterinary drugs from medicated to non-medicated feeds in commercial feed manufacturing plants. *Food Addit. Contam. Part A* 30 (6), 1100–1107.
- Tran, T.H.T., Everaert, N., Bindelle, J., 2018. Review on the effects of potential prebiotics on controlling intestinal enteropathogens *Salmonella* and *Escherichia coli* in pig production. *J. Anim. Physiol. Anim. Nutr.* 102 (1), 17–32.
- World Health Organization, 2017. WHO Guidelines on Use of Medically Important Antimicrobials in Food-Producing Animals. World Health Organization.