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Environmental Research 238 (2023) 117242

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

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A B S T R A C T

The use of antibiotics in food-producing animals can induce the presence of residual substances in manure, which are then released into the environment and may contribute to soil and groundwater contamination. During the on-farm implementation of strategies to improve animal health and welfare in chicken and pig farms, the consequences of antibiotic use were evaluated in terms of the occurrence and levels of antibiotic residues in manure.

A set of 35 broiler farms from Cyprus, Greece, the Netherlands and 40 pig farms from France and Italy provided a total of 350 manure samples. The primary objective was to develop a specific LC/MS/MS method capable of quantifying antibiotic residues in both types of manure. The method was able to detect fifteen antibiotics belonging to nine classes, with validated limits of quantification of 10–20 μg/kg, and accuracies ranging from 81% to 138%.

With the exception of amoxicillin, which was never detected in any manure, all antibiotics used were detected in manure from treated animals with typical concentrations ranging from 10 to 99198 μg/kg for both chickens and pigs. The occurrence of residual antibiotics was higher in chicken than in pig manure, especially for fluoroquinolones and doxycycline which were detected in 89% and 100% of the chicken manure, respectively, and in 28% of the pig manure. The impact of the health plans on the antibiotic load manure was assessed by measuring for each farm the ratio of the sum of all antibiotic concentrations measured after and before the implementation of the plan. The results showed that, in addition to the frequency of treatments, the class of antibiotic used is an important factor to consider as it strongly influences the stability/instability of the compounds, i.e. their ability to persist in the manure of food-producing animals.

1. Introduction

Since the discovery of penicillin in 1928, many antibiotics, both natural and synthetic, have been developed and marketed to kill or stop the growth of bacteria responsible for many infections (Abraham and Chain, 1940). It has been known for several decades that the overuse and/or misuse of antibiotics can lead to the development of antibiotic-resistant bacteria, which pose a global threat to human health, animals and the environment (Ghimpețeanu et al., 2022; Landers et al., 2012; World Health Organization, 2014). In this context, in 2017, the European Commission adopted a new "One Health" action plan to promote new solutions to treat infectious diseases, improve diagnosis and control the spread of antimicrobial resistance, and implemented guidelines to promote the prudent and rational use of antibiotics in veterinary medicine (European Commission, 2017). The development of intensive farming has led to the massive use of antibiotics in food-producing animals. In Europe, the consumption of antibiotic in food-producing animals varies widely between countries, ranging from 4.6 to 423.1 mg/kg of estimated biomass in 2017 (European Centre for Disease Prevention and Control (ECDC) et al., 2021). Tetracyclines, penicillins and sulfonamides were the most sold classes of antibiotics for food-producing animals, accounting for 32%, 26% and 12% of the total veterinary antibiotic sales respectively. At the species level, this high use of antibiotics is mainly due to the collective oral treatment of cattle, pigs and chickens (European Medicines Agency, 2018; Van Boeckel et al., 2015).

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https://doi.org/10.1016/j.envres.2023.117242
Received 9 May 2023; Received in revised form 15 September 2023; Accepted 26 September 2023
Available online 26 September 2023

Following the ban on the use of antibiotics as growth promoters in Europe (European Commission, 2006), mass medication - collective oral treatment - has been identified as a major concern regarding the prudent use of antimicrobials in food-producing animals, with this concern being greater for treatments administered via medicated feed than via drinking water (European Medicines Agency, 2020). Indeed, the use of antibiotic formulations in drinking water is preferred due to their ease of administration and superior dose control. However, it is essential to optimise the solubility of the antibiotic and its dosing regimen to ensure maximum efficacy (Ferran et al., 2020; Ferran and Roques, 2019).

Regardless of how the drugs are administered to the animals, most of the drugs are excreted in their manure, which is often used as fertiliser by being dumped directly on the land surrounding the farms. This common practice allows antibiotic residues in manure to leach into soil and water, potentially contributing to the spread of antibiotic resistance. Many studies have described that the environmental matrices could also be an important source of antimicrobial resistance (Haenni et al., 2022; Zhou et al., 2020). The spread of antibiotics in the environment should also be taken into account when assessing the risk of the emergence of antimicrobial resistance, and requires the development of reliable methods for the quantification of antibiotics in environmental matrices and in particular in manure. Due to the diversity of their physico-chemical properties, antibiotics are mainly quantified according to their class and using liquid chromatography coupled with optical detection (UV or fluorescence) or mass spectrometry and using specific extraction protocols such as solid phase extraction (SPE) or modified quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction (Ajibola et al., 2022; Argüeso-Mata et al., 2021; Guo et al., 2016; Poindexter et al., 2022; Rashid et al., 2020; Van den Meersche et al., 2016; Zhi et al., 2020; Zhou et al., 2012). In general, multi-residue methods have been developed to target about ten antibiotics in the same class and in the most commonly used classes, without any knowledge of the antibiotic treatments used on farms (Zhi et al., 2020). As a result, information on the behaviour of antibiotics in the environment could be missed, firstly because certain antibiotics are not targeted by the analytical method, and secondly because an antibiotic that is not detected in manure does not mean that it has not been administered to the animal previously (Zhi et al., 2020; Zhou et al., 2012).

Our first objective was to develop a specific LC/MS/MS method to reliably quantify antibiotics in broiler and pig manure based on information on the antibiotic treatments applied to the animals prior to manure sampling. This study is part of the EU Horizon 2020 research project Healthylivestock, which aims to propose realistic best practices to reduce the need for antimicrobials while taking into account feasibility, societal acceptability and economic viability ("Healthy Livestock, " 2018). This project evaluated the impact of the implementing tailor-made health plans, including biosecurity, on the presence of antibiotic residues in the manure collected from the farms selected for the experiment. Tailor-made health plans were developed for each farm using a BiosEcurity Assessment Tool (BEAT) to improve animal health and limit the use of antimicrobials. This tool looks at the risk of introducing a pathogen into the farm, the risk of exposing susceptible animals and the risk of spreading disease within the farm (Schreuder et al., 2023; Scollo et al., 2022). The broiler farms were from Cyprus, Greece and the Netherlands, and the pig farms were from France and Italy. This study is, to the best of our knowledge, one of the first to look at the impact of health plans aimed at reducing the use of antibiotics on the spread of antibiotics in the environment.

2. Materials and methods

2.1. Manure collection

Manure samples were collected from seven, fifteen and thirteen broiler houses in Cyprus, Greece and the Netherlands, and from twenty pig farms in France and Italy (40 farms in total). Each farm was selected from on the basis of the involvement of its veterinarians and its antibiotic use practices. For each farm, two samples were collected from two different locations and at least two broiler flock cycles or during the weaning or fattening period for pigs, resulting in 117 and 240 broiler and pig manure samples, respectively, for analysis (Table S1).

Tailor-made health plans were developed for all farms using the same BiosEcurity Assessment Tool (BEAT) developed by Scollo et al. for pig farms and Schreuder et al. for broiler farms (Schreuder et al., 2023; Scollo et al., 2022). This tool consists of a worksheet in Microsoft Excel, instructions for new users and a list of the risks of major animal diseases were included. The tool was structured according to risk zones (low, medium and high) and according to the objectives to be achieved. Manure was sampled at the end of each of the production cycle or period in 40–60 mL in labelled polypropylene vials. The vials were stored at −20 °C and kept frozen until shipment and LC/MS/MS analysis.

2.2. Chemicals

The antibiotics used to develop the method for quantification in broiler and pig manure are listed in the Supplementary Data (Table S2) together with their CAS number, molecular weight (MW) and supplier. The isotopic internal standards (IS) required for the method are also listed in Table S2.

2.3. Preparation of standard and quality control samples

Stock solutions were prepared for each antibiotic by weighing 1 mg of powder into 1 mL of suitable solvent for total dissolution. Working solutions were prepared by serial dilution of the antibiotics in H_2O/AcN (95/5) to give final concentrations of 10000, 1000, 100 and 10 μg/L of each antibiotic. Standard and quality control (QC) solutions were prepared by diluting working solutions in 200 mg of manure to obtain concentrations ranging from 10 to 10000 μg/kg for the standard curve calibrator and at 20, 200 and 2000 μg/kg for the quality control (QC) samples. The internal standard solution was prepared in H_2O/AcN (95/5) at 200μg/L and stored at 4 °C.

2.4. Extraction procedure

Broiler and pig manure was crushed for 30 s with a blender. Two hundred micrograms of the crushed manure were added to 20 μL of internal standard mixture (1000 μg/L) and vortexed for 3 min at 1500 rpm with 200 μL of MeOH acidified with 1% formic acid (FA). The mixture was centrifuged at 4 °C for 10 min at 20000 g. The supernatant was isolated from the pellet which was extracted a second time with 200 μL of MeOH, 1% FA and centrifuged once again. The two supernatants were combined and evaporated to dryness under nitrogen. The dry extract was diluted with 500 μL of H_2O/AcN (95/5), 0.1% FA and filtered through a 0.2 μm nylon membrane.

2.5. LC/MS/MS method

Antibiotic assays were performed using an ultra-high performance liquid chromatography (U-HPLC) system (Nexera LC40) coupled to an 8045 triple quadrupole mass spectrometer (Shimadzu, Japan). The antibiotics were eluted at a flow rate of 0.4 mL min^{-1} on an Acquity BEH C18 (2.1*100; 1.7 μm, Waters, MA, USA) at 40 °C. The mobile phase consisted of water (%A) and acetonitrile (%B), both acidified with 0.1% formic acid. The gradient elution was as follows: t(0 → 0.5 min) 95%A; t (0.5 → 9 min) 95% A → 20 %A; t(9.0 → 9.1min) 5%A; t(9.1 → 10 min) 5%A (back to equilibrium). The injection volume was 10 μL. Each antibiotic was ionised by electrospray and detected using multiple reaction monitoring (MRM) mode. The MRM transitions of each analyte and IS with their corresponding cone voltages and collision energies are provided in the Supplementary Data (Table S3). Chromatographic data were monitored using Labsolutions Insight software (Shimadzu, Japan).

2.6. Performance of the method

The performance of the method was evaluated in terms of linearity, selectivity, inter- and intra-day precision and accuracy. To assess selectivity, six replicates of blank manure were extracted without the use of IS. The calibration curve was determined using seven points containing antibiotics spiked into the manure at concentrations ranging from 10 to 10,000μg/kg. To assess the linearity of the assay, three calibration curves were generated using linear ($Y = aX + b$) and quadratic ($Y = aX^2 + bX + c$) models, with weightings of 1, 1/X, and 1/X^2 (where X is the nominal concentration). The optimal calibration model was selected from: the visual inspection of the residual distribution plot against nominal concentrations, a lack-of-fit test to assess the goodness-of-fit of the model, and the calculation of the relative concentration residuals (RCR%). The LOQ was defined as the lowest concentration level on the calibration curve that could be quantified with an RCR% of less than ±20%. Precision was assessed using the percentage coefficient of variation (CV% = 100* standard deviation/mean), while accuracy was determined as the ratio of the mean measured concentration to the nominal value and expressed as a percentage. Accuracy and precision were assessed using QC samples at three concentration levels (low = 20 μg/kg, medium = 200 μg/kg, and high = 2000 μg/kg) covering the range of concentrations on the calibration curve.

2.7. Evaluation of the impact of the health plans on the antibiotic residues in manures

The environmental impact of these health plans was assessed by measuring antibiotic residues in manure. An indicator ($R_{\Sigma ATB} = \Sigma ATB(Visit\ 2)/\Sigma ATB(Visit\ 1)$) was constructed for each farm, consisting of the ratio ($R_{\Sigma ATB}$) of the sum of the concentrations of all antibiotics quantified in the manure sampled after implementation of the plan (ΣATB (Visit 2)), divided by the sum of the concentrations of all antibiotics quantified in the manure sampled before implementation of the plan (ΣATB(Visit 1)).

If an antibiotic was used on the farm and not detected in the manure, we used in the sum a concentration equal to the lowest LLOQ (i.e. 10 μg/kg) divided by 2 in the sum. Results for antibiotic residues in manure were considered not different between the two visits if $R_{\Sigma ATB}$ was between 0.5 and 2.

3. Results

3.1. Manure collection and treatments

Of the 117 chicken manure samples, 89 were collected after antibiotic treatment, 25 were collected from farms without antibiotic treatment and information three samples were missing information. More manure was collected from the pig farms (240 samples), but many lacked information on antibiotic treatment (160 samples). The other 80 samples for which information was available were collected after antibiotic treatment. Based on the information provided, one to three antibiotics were administered to chickens during a production cycle and three to ten antibiotics were administered to pigs during weaning and/or fattening. The list of antibiotics used on the farms prior to manure collection was provided with the samples. In total, twenty antibiotics were used at least once in an antibiotic treatment, representing many classes of antibiotics (Table 1).

As shown in Table 1, amoxicillin was the most commonly used antibiotic in pig and broiler treatments, accounting for 78% and 100% of manure samples with documented antibiotic treatments, respectively. For broilers, (fluoro)quinolone and tetracycline (doxycycline only) treatments were also predominant with 66% and 57% of manure with documented antibiotic treatments respectively. For pigs, more antibiotics of different families were used, the most common being phenicols (98%), fluoroquinolones (90%) and macrolides (68%) after amoxicillin.

Table 1
Main antibiotics used in chicken and pig farm of the manures collected after treatments.

Antibiotic family	Number of manure samples		% manure with documented ATB treatment	
Antibiotic	Chicken (n = 89)	Pig (n = 80)	Chicken (n = 89)	Pig (n = 80)
Penicillins	**69**	**80**	**78%**	**100%**
Amoxicillin	69	80	78%	100%
(Fluoro) Quinolones	**59**	**72**	**66%**	**90%**
Enrofloxacin	57	20	64%	25%
Marbofloxacin	0	52	0%	65%
Flumequine	2	–	2%	–
Tetracyclines	**51**	**36**	**57%**	**45%**
Doxycycline	51	36	57%	45%
Phenicols	**–**	**78**	–	**98%**
Florfenicol	–	78	–	98%
Macrolides	**2**	**54**	**2%**	**68%**
tylosin	1	34	1%	43%
other	1	20	1%	25%
Other family	**27**	**78**	**30%**	**98%**
lincosamines	6	14	7%	18%
aminosides	6	16	7%	20%
cephalosporines	0	4	0%	5%
diaminopyrimidines	6	8	7%	10%
pleuromutilines	0	12	0%	15%
polymyxines	3	16	3%	20%
sulfonamides	6	8	7%	10%

Doxycycline was also used to a lesser extent, accounting for 45% of pig manure treated with antibiotics. Other antibiotics were occasionally administered but were reported in less than sixteen of the total samples collected for each species. On average, from one to three antibiotics were administered to broilers during a production cycle and, certainly because the cycle is longer than that of broilers, pigs could receive from three to ten antibiotic treatments during the weaning and fattening phases.

3.2. Performance of the LC/MS/MS method

Firstly, the multiple reaction monitoring (MRM) was optimised by infusing working solution at 100 μg/L. Two transitions were optimised for each antibiotic, the first used for quantification and the second for confirmation (Table S3). In a second step, the chromatographic elution was optimised to separate the antibiotic from the matrices with good repeatability and within a few minutes. All the samples were eluted on the Acquity BEH C18 column in less than 10 min (Fig. 1). Several extraction procedures from the literature were tested using either solid-phase extraction (SPE) or dispersive SPE (Guo et al., 2016; Patyra et al., 2020; Rashid et al., 2020; Zhou et al., 2012). The best results for most of the antibiotics were obtained by liquid-solid extraction using acidic MeOH and an additional extraction with ethyl acetate, which increased the extraction yield of TMP, lincomycin, florfenicol, sulfonamides and macrolides from 3 to 40% (Fig. S1).

Conversely to most of methods developed in environmental matrices, the calibration curve, LOQs and QC were assessed directly in the manures. The performance of the method was evaluated for the fifteen antibiotics using calibration points and quality controls (QC) spiked in blank pig manure (Table 2).

However, despite all these developments, we were unable to extract amoxicillin at a concentration lower than 500 μg/kg. In addition, a loss of signal was observed on the chromatograms within 2 h of extraction. The other antibiotics were quantified in the 10-10,000 μg/kg concentration range. The limit of quantification (LOQ), determined at as the first point of the calibration curve, was 10 μg/kg for most of antibiotics except for marbofloxacin, which was 20 μg/kg. LOQ accuracies were evaluated over five days and ranged from 94 to 109% with an inter-day precision CV% of less than 8%. The accuracy and precision of the

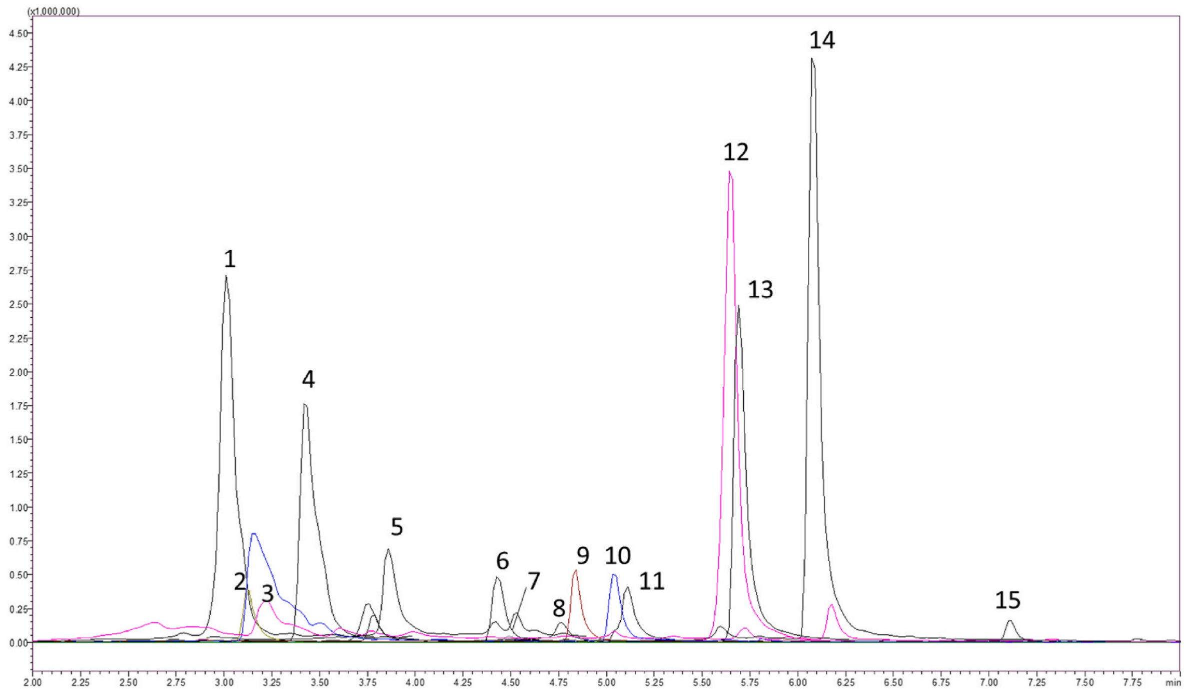

Fig. 1. Total ion chromatogram of antibiotics extracted from pig manure spiked with 50 μg/kg antibiotics (1: Lincomycin, 2: Tildipirosin, 3: Marbofloxacin, 4: Trimethoprim, 5: Enrofloxacin, 6: Sulfamethoxazole, 7: Florfenicol, 8: Doxycycline, 9: Ceftiofur, 10: Sulfadimethoxine, 11: Tilmicosin, 12: Tylosin, 13: Flumequine, 14: Tiamulin, 15: Tylvalosin).

Table 2
Performance of the method for the quantification of fifteen antibiotics in pig manure.

Antibiotic	Calibration range [μg/kg]	Model, weighting	Low concentration QC 20 μg/kg (n = 11)		Mid concentration QC 200 μg/kg (n = 12)		High concentration QC 2000 μg/kg (n = 11)	
			Accuracy %	Precision CV%	Accuracy %	Precision CV%	Accuracy %	Precision CV%
Amox	500–10000	Not stable after extraction from manure						
Enro	10–10000	linear, $1/X^2$	93%	22%	93%	9%	106%	8%
Marbo	20–10000	linear, $1/X^2$	102%	18%	106%	6%	108%	4%
Flume	10–1000	linear, $1/X^2$	136%	25%	104%	6%	Above calibration range	
Doxy	10–10000	linear, $1/X^2$	89%	23%	81%	32%	102%	16%
Flor	10–5000	linear, $1/X^2$	117%	16%	106%	6%	96%	7%
TMP	10–10000	linear, $1/X^2$	130%	12%	106%	4%	104%	4%
SDM	10–10000	linear, $1/X^2$	138%	10%	112%	8%	107%	7%
SMX	10–10000	quadratic, $1/X^2$	121%	8%	108%	7%	106%	8%
Linco	10–1000	quadratic, $1/X$	103%	7%	95%	6%	Above calibration range	
Tilmi	10–10000	linear, $1/X^2$	124%	40%	108%	12%	100%	16%
Tylo	10–5000	linear, $1/X^2$	114%	12%	119%	14%	95%	17%
Tylva	10–5000	linear, $1/X^2$	123%	27%	116%	12%	101%	16%
Tildi	10–10000	linear, $1/X^2$	101%	24%	99%	12%	120%	24%
Ceft	10–5000	linear, $1/X^2$	101%	11%	108%	6%	109%	6%
Tia	10–1000	linear, $1/X^2$	112%	6%	103%	18%	Above calibration range	

method were evaluated using three QC samples over five days. The results presented in Table 2 demonstrate the suitability of the method for the determination of antibiotic residues in manure.

3.3. Residual antibiotics in broiler manures

A total of 117 samples of broiler manure from Cyprus, Greece and the Netherlands were analysed and the results in terms of detection frequencies and concentration ranges for each antibiotic are reported in Table 3.

Doxycycline and enrofloxacin were the most commonly used antibiotics in broiler treatments, being detected in 54% percent of the manures, with concentrations ranging from 44 to 99198 μg/kg and 14–6033 μg/kg respectively. The other antibiotics were detected in less than 10% of the samples.

A comparison between the number of broiler manure samples treated with antibiotics and the number of manure samples in which antibiotics were detected is shown in Fig. 2. With the exception of amoxicillin, which was never detected in manure due to its poor stability, the antibiotics were found in more than 50% of the manures from treated broilers and up to 100% for doxycycline, tilmicosin and flumequine. It is noteworthy that some antibiotics were detected in broiler manure samples without any documented use of these treatments. This was the case for 10 samples tested positive for doxycycline, 25 for enrofloxacin and to a lesser extent for TMP and sulfonamides.

3.4. Residual antibiotics in pig manures

A total of 240 samples of pig manure from France and Italy were analysed, and the results in terms of detection frequencies and

Table 3
Frequency and concentration ranges of antibiotics detected in broiler and pig manure.

Antibiotic	Broiler (n = 117)		Pig (n = 240)	
	Detection frequency %	Concentration range [μg/kg]	Detection frequency %	Concentration range [μg/kg]
Amox	nd	nd	nd	nd
Doxy	54%	44–99198	8%	24–16799
Enro	54%	14–6033	5%	13–97
TMP	8%	13–197	5%	16–2765
Tilmi	1%	15	19%	12–18930
SMX	4%	19–156	1%	48–298
Linco	4%	10–122	2%	12–304
Tylo	4%	114	2%	10–69
Flume	8%	839–1555	1%	10–68
Tylva	na	na	1%	17–116
Marbo	na	na	3%	27–21742
SDM	na	na	4%	10–1384
Ceft	na	na	0%	268
Tia	na	na	2%	31–326
Tildi	na	na	0%	nd
Flor	na	na	2%	44–1355

nd: not detected; na: not analysed.

concentration ranges are presented in Table 3 for each antibiotic.

Fewer antibiotics were detected in pig manure than in broiler manure with a detection frequency of less than 20%. Tilmicosin was the most frequently detected antibiotic in pig manure with a detection frequency of 19% and a concentration ranging from 12 to 18930 μg/kg, mainly due to the larger number of manures collected in France (n = 160). To a lesser extent, doxycycline and fluoroquinolones (enrofloxacin and marbofloxacin) were also found in some pig manure, with detection frequencies of 8%, 5% and 6%, respectively. The concentration ranges were in the same order of magnitude, 24–16799 μg/kg for doxycycline and 27–21742 μg/kg for marbofloxacin. In broilers, the wide range of concentrations measured in manure depended on both the antibiotic and the sample.

A comparison between the number of manure samples from pigs treated with antibiotics and the number of manure samples in which antibiotics were detected is shown in Fig. 3.

Doxycycline and fluoroquinolones were the most commonly detected antibiotics in manure samples from antibiotic-treated pigs, with detection rates ranging from 25% to 28%. However, these detection rates were significantly lower than those found in broilers, where detection rates ranged from 89% to 100% (Fig. 2). It is worth noting that florfenicol, which was present in 98% of the documented treatments, had a detection rate of only 6%. Finally, similar to broilers, several antibiotics were found in the manure of non-treated pigs.

3.5. Evaluation of the impact of the health plans on the antibiotic residues in manures

The impact of the Health Plan on the antibiotic load of manure using the $R_{\Sigma ATB}$ ratio is shown in Table 4 for each country.

$R_{\Sigma ATB}<0.5$ means that the sum of antibiotic residues detected after the Health Plan intervention was lower than before the intervention. If $R_{\Sigma ATB}$ is between 0.5 and 2, no difference with the Health Plan was observed and $R_{\Sigma ATB}>2$ means that the sum of antibiotic residues after intervention was higher than before the intervention.

The majority of the broiler farms did not show a significant difference in antibiotic residues between the two visits. These results were in line with the amounts of antibiotics used between the two visits. In fact, with the exception of Cyprus, the implementation of the health plan in broiler farms did not allow a reduction in the use of antibiotics (Schreuder et al., 2023). Animals were either treated with persistent antibiotics at both visits (Greece) or, conversely, no antibiotic treatment was used at both visits (Netherlands). In general, the broiler farms that were positively influenced by the Health Plan, i.e. where $R_{\Sigma ATB} < 0.5$, reduced their antibiotic residues mainly because the antibiotic treatment was stopped at the second visit or replaced by amoxicillin, which is not detectable in the manure. Conversely, the few farms that did not improve with the Health Plan changed their antibiotic treatments by using more persistent antibiotics, resulting in higher antibiotic levels in manure even though overall use was reduced. This was the case on farms where Tylosin or TMP Sulfonamide was replaced by Flumequine (Netherlands).

As little information on antibiotic use was included in the health plan for the majority of pig farms (n = 160, Table S1), it is difficult to assess the impact of the plan on antibiotic use. However, a significant parameter influencing the impact of the health plan on antibiotic residues in manure was the predominant concentration of tilmicosin. In the

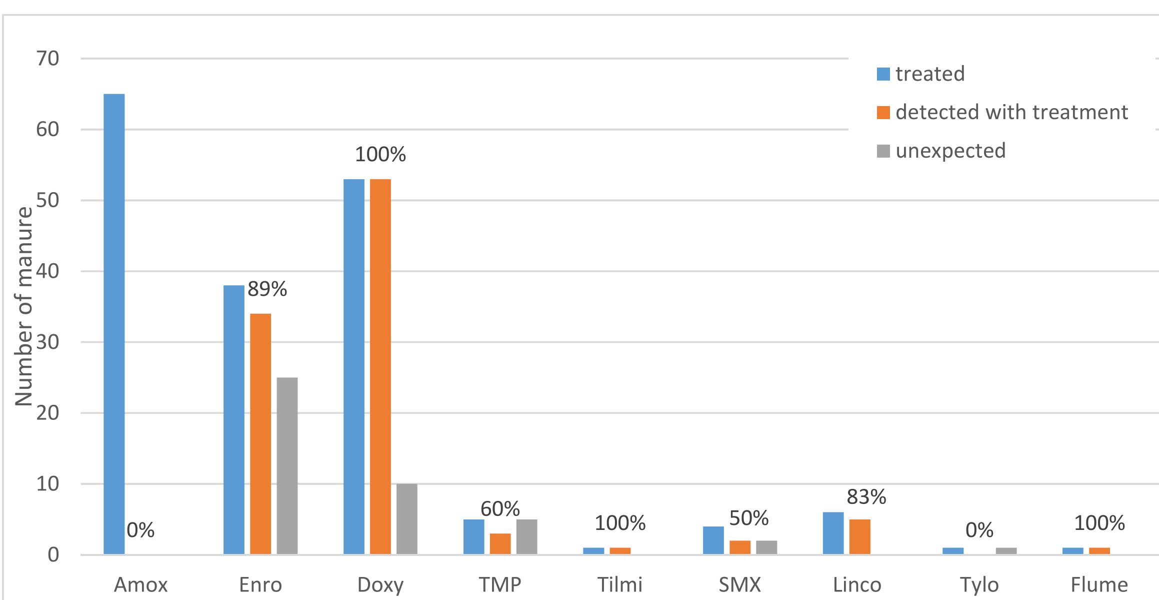

Fig. 2. Number of manure samples from antibiotic-treated broilers (n = 89, blue), number of manure samples in which the corresponding antibiotic was detected and percentage of detection (orange) and number of manure samples from non-treated broilers in which the corresponding antibiotic was detected (unexpected, grey).

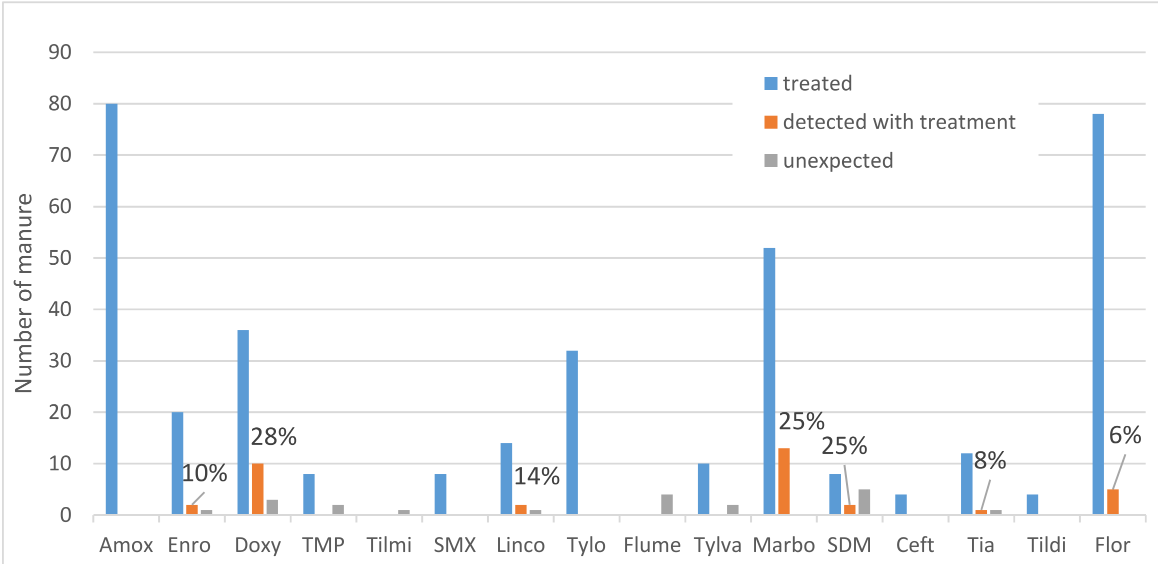

Fig. 3. Number of manure samples from antibiotic-treated pigs (n = 80, blue), number of manure samples in which the corresponding antibiotic was detected and percentage of detection (orange) and number of manure samples from non-treated broilers in which the corresponding antibiotic was detected (unexpected, grey).

Table 4
Overall impact of health plan implementation on the antibiotic residues in manure.

Country	Farms	Nb farms $R_{\Sigma ATB}<0.5$	Nb farms $0.5<R_{\Sigma ATB}<2$	Nb farms $R_{\Sigma ATB}>2$
Cyprus	Broiler	4	3	0
Greece	Broiler	3	11	1
Netherlands	Broiler	0	8	4
Italy	Pig	12	3	5
France	Pig	8	4	8

Italian pig farms, antibiotic concentrations in manure were reduced on 12 farms between the two visits, confirming the slight trend towards a reduction in antibiotic use after the implementation of the health plan on these same farms (Scollo et al., 2022). As for the broilers, this difference in antibiotic concentration could also be attributed to the type of antibiotic used during the two visits and could potentially explain both the improvement or, conversely, the lack of effectiveness of the health plan in reducing antibiotic use. However, in contrast to the broiler manure, a difference in the concentration of persistent antibiotics such as doxycycline or marbofloxacin was also observed between the two visits, despite the fact that the animals received the same treatment at both visits. This observation suggests that either the sample collection was not homogeneous enough compared to the broiler sample collection, or a more effective approach to manure removal and storage was implemented during the second visit. It is therefore recommended to develop a specific tailor-made plan to improve this waste management, which can be an important source of infection in pigs (Scollo et al., 2022).

4. Discussion

The first objective of this study was to develop a method for the simultaneous quantification of veterinary antibiotics from different classes in pig and broiler manure. LC/MS/MS is the method of choice for the determination of small molecules with different physico-chemical properties (Log P, Pka), such as antibiotics, in complex and heterogeneous matrices. As antibiotic treatments were documented for most of the samples, we performed a targeted analysis using the MRM mode on a triple quadrupole mass spectrometer. This system is currently the most effective for the analysis of antibiotic residues in environmental matrices (Rashid et al., 2020). The main challenge of the study was to develop a protocol to extract a wide range of antibiotics from different classes and heterogeneous matrices in order to detect them both at residual levels, i.e. in the μg/kg range, and at a wide range of concentrations when the manure were collected at the end of the treatments. Our method has been successfully validated for fifteen antibiotics from nine different classes, using a 2 or 3-log concentration range for calibration, with validated limits of quantification of 10–20 μg/kg These LOQs were either better than those reported in some recent similar studies (Ajibola et al., 2022; Patyra et al., 2020) or in the same range but with a simpler extraction procedure (Poindexter et al., 2022). Protocols for the quantification of 40 antibiotics from ten classes have already been described with narrower calibration ranges (1–500 μg/kg) and lower limits of quantification (0.01–6 μg/kg) (Guo et al., 2016; Rashid et al., 2020). However, the comparison of our method with these multiclass antibiotic quantification methods is challenging due to differences in the evaluation procedures. In fact, the performance of our method was evaluated using samples spiked directly into manure rather than using a solvent calibration curve; it allowed a realistic quantification of antibiotics over a wider calibration range by taking into account the matrix effect at all concentration levels and did not require the application of a correction factor with the recovery rate. The same applies to the evaluation of the LOQ, which is lower when it is estimated on the basis of the instrumental limit of quantification and corrected by the recovery rate (Guo et al., 2016; Rashid et al., 2020) than when the limits are evaluated directly using LOQs prepared in matrix (Patyra et al., 2020; Poindexter et al., 2022). Moreover, while using matrix-matched calibration curve and QCs, we were unable to extract amoxicillin at concentrations below 500 μg/kg and the extract was not stable for more than 2 h. This phenomenon has been observed not only with LC/MS methods developed in manure (Patyra et al., 2020), but also in *in-vitro* experiments conducted on pig manure at room temperature. These experiments revealed that amoxicillin was degraded within 2 h during the mixing and pretreatment process of the study, leading to a rapid β-lactam ring opening of amoxicillin into amoxicillin-penicilloic and penilloic acids (Liu et al., 2018). Nevertheless, the method remains suitable for the quantification of other antibiotics in manure, with good accuracies (81%–138%) over a wide concentration range (10–10000 μg/mL).

To the best of our knowledge, this study is the first to report on the occurrence of antibiotics in over 350 manure samples collected from five countries, together with information on the treatments administered prior to sample collection for half of the samples. In this specific European campaign, amoxicillin was the most commonly used antibiotic in broiler and pig farms. However, it was not detected in any of the manure samples due to the limitations of the analytical method, which did not allow the quantification of amoxicillin at concentrations below 500 μg/kg. In addition, the low stability of the amoxicillin extracted from the manure made it impossible to analyse. Other factors, such as solubility problems in drinking water and pharmacokinetic behaviour, may also contribute to the lack of detection of amoxicillin in manure (Filippitzi et al., 2019). Fluoroquinolones and doxycycline, mainly used after amoxicillin, were frequently found in the manure samples, with typical concentrations in the mg/kg range for both types of farms. These results are consistent with the presence of veterinary antibiotic residues detected in manure from other European countries (Argüeso-Mata et al., 2021; Karcı and Balcıoğlu, 2009; Scaria et al., 2021; Topi and Spahiu, 2020) and from Asia (Sarker et al., 2020; Wei et al., 2016, 2019; Zhao et al., 2010). Our study shows a higher incidence of antibiotic residues in broiler manure compared to pig manure. Specifically, the incidence of fluoroquinolones and doxycycline in broiler manure from antibiotic-treated animals was 89% and 100%, respectively, compared to 28% and 25% in pig manure from antibiotic-treated animals. It is important to note that the broiler manure collected consisted mainly of a combination of faeces, feathers, and litter, whereas the pig manure consisted mainly of faeces. Indeed, fluoroquinolones and tetracyclines are known to have a high affinity for organic matter, such as that found in broiler manure (Aristilde and Sposito, 2013; Yang et al., 2021). Moreover, Gajda et al. (2019) have recently demonstrated that doxycycline can be detected at high concentrations in broiler feathers during a long post-treatment period. In addition, manure management on pig farms proved to be one of the most important improvements (9.2%) in preventing contamination (Scollo et al., 2022). Taken together, these data may help to explain both the higher frequency and the ubiquitous presence of fluoroquinolones and doxycycline in broiler manure. Macrolides, and in particular tilmicosin, were found in 19% of the 240 pig manures, especially in manures where information on antibiotic treatment was missing. This antibiotic is widely used in European livestock, but few studies have addressed its occurrence in manure and environmental persistence. This antibiotic is mainly excreted in large quantities in faeces after collective treatments and could be released in the faeces for more than 23 days at mg/kg levels (Perruchon et al., 2022). Conversely, florefenicol is mainly excreted in the urine (76% of the oral dose) and to a lesser extent in the faeces (European Medicines Agency, 1999). In most farms, pigs are typically housed on gratings where urine is separated from faeces. This may help to explain why florfenicol has been poorly detected in pig manure (6%), and at low concentrations, despite its large use (98% of the documented treatments). In general, with the exception of tilmicosin, the occurrence of antibiotics in manure after antibiotic treatment was much lower in pig manure than in broiler manure. This difference in occurrence confirms the better manure management highlighted in these pig farms (Scollo et al., 2022) than in broiler farms where no intervention on manure was achieved (Schreuder et al., 2023).

The second objective was to evaluate the impact of the implementation of health and biosecurity plans tailored to each farm on the antibiotic load of manure (Schreuder et al., 2023; Scollo et al., 2022). For this purpose, we built a summary indicator that included the different antibiotics used in the farm and their concentrations when detected in the manure. The indicator and its analysis were designed for comparison within farms, i.e. the initial level of antibiotic load was not taken into account. We felt that this was the only way to provide a comprehensive and quantitative view of the heterogeneous situations associated with the implementation of tailor-made farm-specific health plans. It should be noted that the implementation of these health plans was associated with a reduction in antibiotic concentrations in meat and water, sampled from the same farms (Gajda et al., 2023). However, the analysis of the impact of these health plans on the antibiotic load of manure showed contrasting results, probably related to the fact that this phenomenon does not only depend on the amount of antibiotics used. Indeed, if the improvement in biosecurity and health may be the cause of the reduction in antibiotic treatments, the effect of such a reduction on the antibiotic load in manure may be influenced by other factors, the most important of which is probably the stability of the antibiotic, which determines its persistence in the complex matrix of manure. This is probably the reason for the observations from the Netherlands, where the health plans led some farms to replace one antibiotic with a more stable one. In this context, the high stability of fluoroquinolones and tetracyclines in environmental matrices explains their higher presence (frequency and concentration range) in manure samples. Such environmental persistence, which can lead to secondary contamination of soil and groundwater, could probably explain the presence of doxycycline in some manure samples from untreated animals (Scaria et al., 2021). Further experiments, such as groundwater sampling, should be carried out to investigate this phenomenon. Finally, the characteristics of the antibiotic, as well as the time frame of the observations, are important conditions for achieving a reduction of antibiotic contamination in manure (and subsequently in the environment). To improve the positive effects of health plans on the reduction of antibiotic use, strategies to remove antibiotics from manure before spreading should be included in the management of the manure according to the type of antibiotic use (Gaballah et al., 2021).

In conclusion, this study developed a successful LC/MS/MS method for the quantification of veterinary antibiotics of different classes in pig and broiler manure, detecting fifteen antibiotics from nine different classes detected at acceptable limits of quantification. Amoxicillin, the most commonly used antibiotic in pig and broiler farms, was not detected in any of the manure samples from these animals. In addition, we observed that fluoroquinolones and doxycycline are prevalent in manure samples, particularly in broiler manure, and that manure management and farm design could also influence the antibiotic occurrence. Our evaluation of the health plans tailored to each farm indicates that, in addition to the frequency of treatments, the class of antibiotic used is an important factor to consider, as it strongly influences the stability/instability of the compounds, i.e. their ability to persist in the manure of food-producing animals. These results underline the need to a develop tailor-made plan, taking into account both the type of antibiotic use and the farm design, in order to improve manure management and limit the spread of antibiotics in the environment.

Funding

This study was conducted as a component of the Healthy Livestock project, which was funded by the European Union's H2020 research and innovation program through grant agreement number 773436.

CRediT authorship contribution statement

Marlene Z. Lacroix: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Felipe Ramon-Portugal:** Methodology, Validation, Formal analysis, Investigation. **Alicia Huesca:** Methodology, Validation, Investigation, Formal analysis. **Kyriacos Angastiniotis:** Conceptualization, Investigation, Resources, Writing – review & editing. **Maro Simitopoulou:** Conceptualization, Investigation, Resources. **George Kefalas:** Conceptualization, Investigation, Resources, Writing – review & editing. **Paolo Ferrari:** Conceptualization, Investigation, Resources, Writing – review & editing. **Pierre Levallois:** Conceptualization, Investigation, Resources. **Christine Fourichon:** Conceptualization, Investigation, Resources, Writing – review & editing. **Maaike Wolthuis-Fillerup:** Conceptualization, Investigation, Resources, Writing – review & editing. **Kees De Roest:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing –

review & editing. **Alain Bousquet-Mélou:** Conceptualization, Formal analysis, Investigation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117242.

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