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ANTIMICROBIALS IN ANIMAL FAECES (CHICKEN, COW AND SWINE) FROM THE BREEDING FARMS IN THE UPSTREAM OF SAI GON RIVER

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Abstract. The widespread use of antimicrobials in husbandry has increased antimicrobial residues in animal faeces. Therefore, the investigation of antimicrobials in animal faeces is essential because they are the main cause of environmental pollution. This study analyzed and assessed samples of chiken, cow and swine faeces for seven antimicrobials, namely trimethoprim (TRI), ormethoprim (ORM), ofloxacin (OFL), norfloxacin (NOR), tetracycline (TET), chlortetracycline (CTE) and tylosin (TLS). Samples were taken from faeces heaps at breeding farms in the upstream of the Sai Gon river. The samples were analyzed in the laboratory by using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine antimicrobials. TRI, with the highest mean concentration, was found in all fecal samples (10,399 - 47,987 μg/kg) while ORM with the lowest average concentration was only detected in chicken fecal samples (542 μg/kg). CTE, with the second high mean concentration, was also found in all fecal samples (1,085 - 12,385 μg/kg), whereas TET was only detected in chicken fecal samples (9,050 μg/kg). OFL with the third average concentration, was aloso found in all fecal samples (1,516 - 9280 μg/kg), while NOR was detected in cow fecal samples at a low mean concentration (2,479 μg/kg). TLS was only found in chicken and cow fecal samples (798 - 5,802 μg/kg). These numbers show a very large amount of consumption of antimicrobials in livestock production. The results from this study are likely to encourage further monitoring of antimicrobials used in livestock production, and promote discussion of existing policies regarding the inclusion of antimicrobials in animal feed rations and veterinary medicines.

Keywords: antimicrobial, faeces, Sai Gon river, concentration, livestock.

Classification numbers: 3.2.1, 3.3.1, 3.3.2

1. INTRODUCTION

Recently, the presence of antimicrobials in the environment has received more and more attention worldwide due to its risk not only from an environmental perspective but also affecting

human health. Veterinary antimicrobials are chemical drugs used in livestock to treat diseases, prevent infections, promote growth, or improve feed efficiency. After being imposed on livestock, these pharmaceuticals are typically absorbed through animals' digestive and circulatory systems and excreted through their excretory system. The pharmaceuticals are generally metabolized and deactivated for biological functions after passing through the animal body. A significant portion (10 - 90 %) of the applied quantities, however, may remain intact as parent compounds and deposit in animal tissues and excrement [1]. Jacobsen and Halling-Sørensen, (2005) [2] detected tetracyclines and sulfonamides in swine faeces, but no tylosin was detected because of its poor recovery from faeces. Tetracyclines (TCs), macrolides (MLs), and fluoroquinolones (FQs) are the most frequently detected antimicrobials and exhibited a broad concentration range (frequently at mg/kg level) in animal faeces and agricultural soils of some European countries [3]. However, there is little information on antimicrobials in chicken, cow, and swine faeces in Vietnam. Therefore, we conducted analysis and evaluation of the residues of trimethoprim, ofloxacin, ormetoprim, norfloxacin, tetracycline, chlortetracycline and tylosin in fecal samples. The selected antimicrobials are commonly used in the veterinary medicines in Cu Chi district, Ho Chi Minh City (HCMC) - a typical breeding region in the upstream of the Sai Gon river.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Diaminopyrimidines (DMs): trimethoprim (TRI) and ormethoprim (ORM); *Fluoroquinolones (FQs):* ofloxacin (OFL), norfloxacin (NOR); *Tetracyclines (TCs):* chlortetracycline (CTE), tetracycline (TET), and *Macrolides (MLs):* tylosin (TLS) were purchased from Sigma-Aldrich (Viet Nam). All standards such as trimethoprim-d3 (TMP-d3), tetracycline-d6 (TC-d6) were obtained from Sigma-Aldrich with a purity level higher than 95 %. Deuterium Compounds Performance Reference Substances (PRC) labeled atrazine-desisopropyl (DIA-d5) were purchased from Sigma-Aldrich with a purity level higher than 99 %. The solvent: Methanol (MeOH) (VWR), Acetonitrile (ACN) (Merck) were used to qualify for the LC-MS/MS. Formic acid (99 %), Orthophosphoric acid (85 %), NaOH (50 %) were purchased from Sigma-Aldrich. Ultrapure water (Up-water) was dispensed from an Elga Purelab water purification system. OASIS HLB extraction column (200 mL, 6 mL), Polyethersulfone (PES) (0,1 µm, 90 mm) were supplied by Pall Corporation (Ann Arbor Michigan, USA).

The individual standard solution is diluted at 1 mg/mL in MeOH, for NOR and OFL were added NaOH 1 M to increase the solubility at $1‰$, these stock solutions were stored in amber glass vials at -18 ^{o}C. Then a mixture of standards called the source solution consisting of all the standard mixtures at a concentration of 1 µg/mL was diluted in a mixture of MeOH/Up-water (5/95, v/v). This source solution was diluted to different concentrations at 200 - 100 - 50 - 10 and 5 ng/mL to run a baseline on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS, Agilent 1200 RRLC). These diluted solutions were stored at 4 ^{o}C and renewed after two weeks.

2.2. Methods

2.2.1. Sampling area

This study was conducted in Cu Chi district, HCMC - the upstream of the Sai Gon river. There are two areas of breeding farm in Phuoc Hiep ward (PH) - and An Phu ward (AP) having completely different characteristics (Figure 1). PH is a typical cattle breeding area including cow, swine and buffalo farms, etc. while AP is featured by poultry breeding farms such as chicken. Three cow fecal samples and three swine fecal samples were taken in PH, three samples of chicken faeces were selected from AP farms. At the time of sampling, some farms were spreading epidemics on animals.

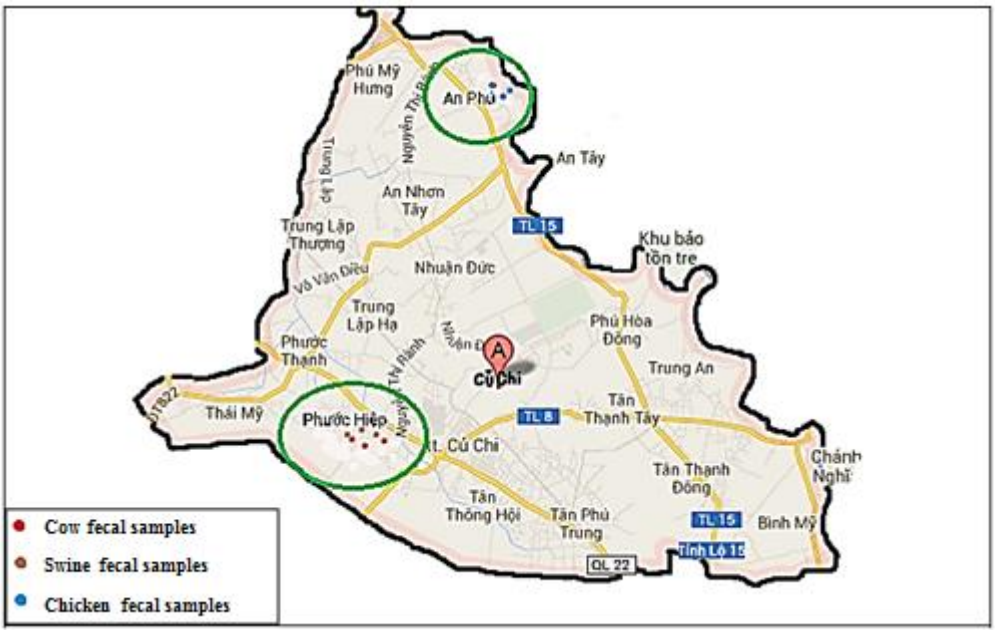

Figure 1. Location of the fecal sampling in An Phu and Phuoc Hiep Ward, Cu Chi District, HCMC

This study focuses on surveying types of antimicrobials and then selecting relevant antimicrobials based on the following criteria: dosages, purposes of usage (medicine, animal husbandry, aquaculture), frequencies of occurrence, environmental sustainability and finally, exploring capacity of laboratory [4].

2.2.2. Sampling collection

For fecal samples, at each sampling point, about 100 grams of faeces were taken and mixed with a plastic spoon placed in a zip bag and stored at 4 ^{o}C before being transfered to the laboratory. At the CARE-Rescif laboratory, Ho Chi Minh University of Technology (HCMUT), fecal samples were dehydrated by lyophylization, then crushed and sieved through a 1 mm sieve to remove impurities and then extracted by ultrasonic waves to obtain antimicrobials.

2.2.3. Sample preparation and analysis

For fecal samples, 2grams of dried samples were placed in a 30 mL glass tube and extracted twice (the first time under acidic conditions and the second time under basic conditions) with 10 mL (MeOH/1 M H_3PO_4 80/20 v/v, + 0.1 % EDTA 1 M), then 10 mL (MeOH/5 % NaOH 20/80 v/v, 0.1 % EDTA 1M). Two extraction solutions were mixed together and blown with N_2

at 40 ^{o}C until a few mL of volume remained. This concentrated solution was added to 200 mL of Milli-Q water and adjusted to pH = 7 with orthophosphoric acid (50 %). A set of subsamples was then stored at 4 ^{o}C and analysed daily over the course of a week. Meanwhile, another set of sub samples was stored at -18 ^{o}C and analysed weekly over a one-month period.

Then solid-phase extraction was conducted prior to analysis by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) described by Tuc *et al.* (2011) [5]. The HPLC system consists of: a separation module with a Agilent 1200 RRLC binary pump (600 bar), a drain system (degasser), and an automated sample collection and injection system. The substance after being isolated from the HPLC system was determined by an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and operating in positive mode. MS/MS data were acquired as MS/MS signal acquisition performing in Selected Reaction Monitoring (SRM) mode (Table 1). Argon (99.9 %) was used as a collision gas while nitrogen was used as a carrier gas (11.0 L/h). The source temperature is 300 ^{o}C. The parameters of MS/MS: fragmentation and collision energy (CE) were performed by an Agilent Optimized software. Separation was achieved at 35 ^{o}C and the total run time was 12.5 min. During MS/MS analysis, the sample injection loop was flushed as follows to prevent cross-contamination: 100 % UP-water for 2 min, 100 % MeOH for 4 min (0.5 mL/min). Note that two runs were performed for each sample: in the first series of analyses, the pH of the sample was adjusted at pH 4, while in the second series, it was adjusted at pH 7.

Table 1. Acquisition parameters for anitimicrobial analysis in SRM mode. Frag: fragmentor; CE: collision energy [5]

	Precursor ion (m/z)	Production ion (m/z) quantification	Frag (V)	CE (eV)	Production ion (m/z) quantification	Frag (V)	CE (eV)
TRI	291.2	123.1	145	24	261.1	145	24
ORM	275.2	123.1	145	21	259.2	145	25
NOR	320.1	276.2	135	12	233.1	125	24
OFL	362.2	318.2	140	16	261.1	140	24
TET	445.2	410.2	165	16	154.1	165	24
CTE	479.1	444.1	140	28	462.1	140	12
TLS	916.5	174.1	185	40	772.5	185	28

3. RESULTS AND DISCUSSION

The survey results show that more than 30 types of antimicrobials were used in livestock with completely different dosages and frequencies between farms in which tylosin, trimethoprim, ormethoprim, chloramphenicol, norfloxacin, ofloxacin, enrofloxacin, gentamycin, tetracycline, oxytetracycline, chlortetracycline, and ampicilline were used commonly. All surveyed farms used antimicrobials empirically, not following instructions by a veterinarian. Nhung *et al.* (2016) [6] indicated that, excluding feed, farmers administered 46 mg of different antimicrobial compounds per kg of live pigs and 52 - 276 mg per kilogram of live chickens per year. In this study, we selected a total of seven typical antimicrobials including trimethoprim,

ofloxacin, ormethoprim, tetracycline, norfloxacin, chlortetracycline, and tylosin to analyze and assess their concentrations in chicken, cow, and swine fecal samples.

Antimicrobial concentration in chicken fecal samples

In general, it could be stated that trimethoprim was found in all fecal samples but different residues (Figure 2). Trimethoprim was found in the highest concentration, with a mean of 47,987.667 µg/kg faeces. In 3 chicken fecal samples, trimethoprim was detected to be 87,692 µg/kg faeces, 54,326 µg/kg faeces and 1,945 µg/kg faeces in chicken No.1, No.2 and No.3 samples, respectively. Similarly, ormethoprim, ofloxacin, and tetracycline were found at rather high concentration in chicken No.1 and chicken No.2 samples, while chicken No.3 sample show the presence of only chlortetracycline and tylosin at low levels. Tetracyclines, with their broad spectrum of action, are one of the most frequently-used antimicrobials in the poultry industry, not only for prophylaxis or antibacterial therapy but also as growth promoters [7]. However, the tetracyclines are poorly absorbed by the animal, and their active residues can concentrate in meat, urine, and faeces. They are often used for disease prevention in many poultry exporting countries, such as Brazil and China [8]. An analysis of commercial chicken feed formulation available in Viet Namhas revealed that it may contain up to 48.6 % antimicrobials, the most common of which is chlortetracycline (11.4 %) [9]. In addition to poor antimicrobial absorption by the animal, a large number of chickens may be widely infected with diseases, increasing amounts of veterinary medicines. Thus, some antimicrobials in this study were highly detected.

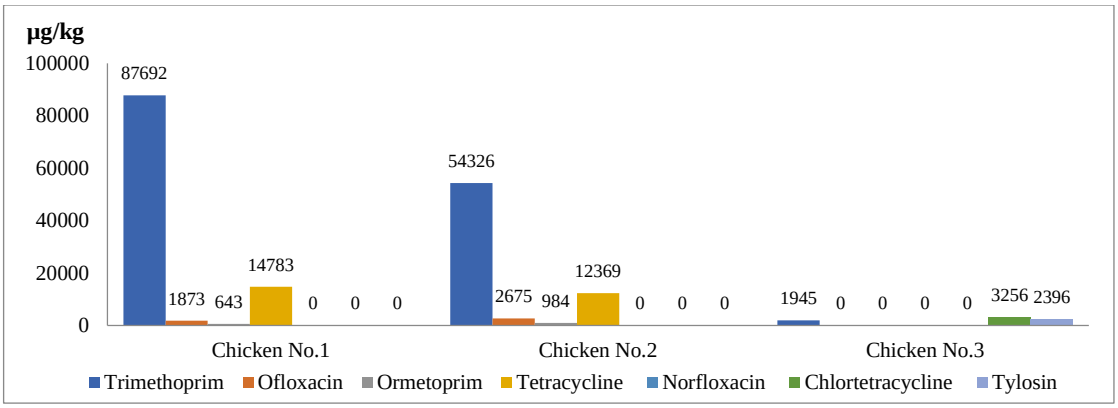

Figure 2. Antimicrobial residues in chicken faeces.

Antimicrobial concentration in cow fecal samples

Figure 3 shows that antimicrobial contents and types are different among three cow fecal samples. Trimethoprim, with the highest average concentration, was found in a mean concentration of 47987.667 µg/kg faeces. In 3 cow fecal samples, trimethoprim was detected at concentratrions higher than 87,000 µg/kg, 54,000 µg/kg and 1,900 µg/kg for cow No.1, cow No.2 and cow No.3, respectively. Chlortetracycline was found in the second mean concentration (9054,333 µg/kg faeces). Ofloxacin, with the third highest average concentration, was found in a mean concentration of 7,405 µg/kg faeces. Norfloxacin and tylosin had a mean concentration of 5,802 µg/kg faeces and 2,479.667 µg/kg faeces, respectively. International Office of Epizootics (2015) [10] reports that a range of antimicrobials is used in the cattle production industry, including aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, glycolipids,

ionophores, macrolides, quinolones, streptogramins, sulfonamides, and tetracyclines. In North America, the major antimicrobials administered sub-therapeutically to beef and dairy cattle with quite high excretion rates are approximately 90 % for sulfamethoxazole, 65 % for chlortetracycline, and, depending on the form of medication, between 50 and 100 % for tylosin [11]. In Europe, macrolides is also commonly used to treat a range of common infections in cattle such as mastitis, foot lesions, respiratory and genital infections [12]. In this study, the regular appearances of trimethoprim, ofloxacin, chlortetracycline and tylosin are consistent with the investigation results of antimicrobial usage in cow breeding farms.

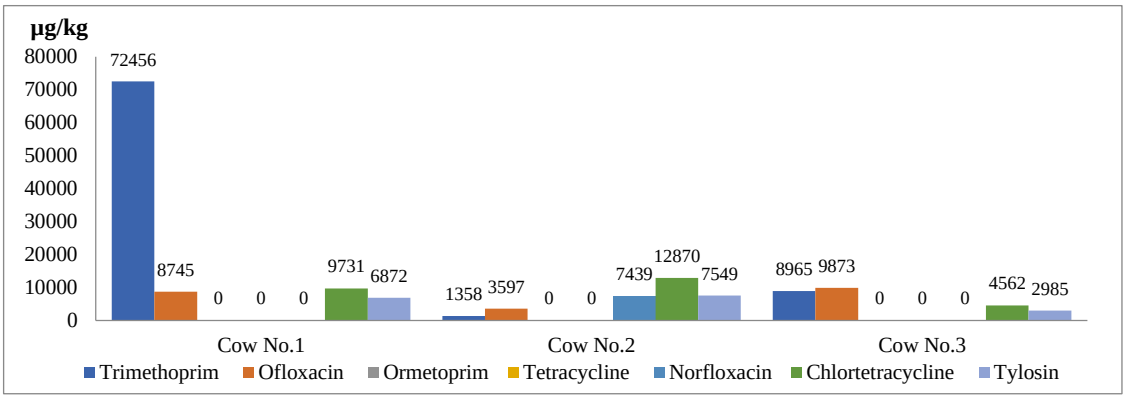

Figure 3. Antimicrobial residues in cow faeces.

Antimicrobial concentration in swine fecal samples

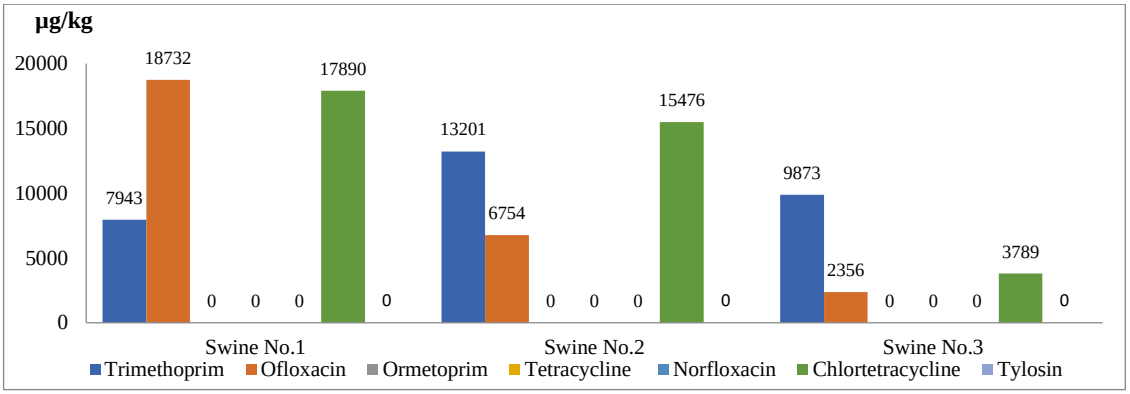

Figure 4. Antimicrobial residues in swine faeces

Figure 4 shows the antimicrobial content in swine fecal samples in three breeding farms with the frequent appearances of trimethoprim, ofloxacin, and chlortetracycline. Chlortetracycline was found in the highest concentration, with a mean of 12,385 µg/kg faeces. In 3 swine fecal samples, chlortetracycline was detected at concentrations higher than 3,700 µg/kg, 15,000 µg/kg and 17,000 µg/kg for swine No.1, swine No.2 and swine No.3, respectively. Trimethoprim was found in the second highest mean concentration (10,339 µg/kg faeces). Ofloxacin, with the third highest average concentration, was found in a mean concentration of 9,280 µg/kg faeces. It is estimated that 286.6 mg of in-feed antimicrobials are used to raise 1 kg

of live pig [9]. An analysis of commercial pig feed formulation available in Viet Namhas revealed that it may contain up to 55.4 % antimicrobials, with the most common to be chlortetracycline (23.9 %) [9]. Results show that these antimicrobials are mainly used in the treatment of swine diseases. According to the analytical data, the concentrations of antimicrobials in swine No.1 and No.2 samples are relatively high compared with swine No.3 samples.

Trimethoprim, ofloxacin, chlortetracycline, and tylosin were found in all fecal samples. Trimethoprim is a broad spectrum antimicrobial with excellent activity against most gram negative organisms and against Staphylococci in the skin. Trimethoprim is generally prescribed for once or twice a day use. It may be given with or without food and should be stored at room temperature. It costs relatively less compared to other antimicrobials, which makes it a popular choice. Tetracycline, norfloxacin and ormethoprim were only found in chicken fecal samples. Tetracylines (TCs) group was also detected in every sample. Tetracycline, with the high average concentration, was only found in chicken faeces (9,050.67 µg/kg faeces) but not detected in cow and swine faeces, whereas chlortetracycline was detected in chicken, cow, and swine faeces with the average concentrations of 1,085.33 µg/kg, 9,054.33 µg/kg and 12,385 µg/kg, respectively. Tetracyclines concentration in swine, chicken, and cow fecal samples ranged from 750 µg/kg to 22,340 µg/kg as reported by Li Yan-Xia *et al.* (2012) [13]. Ling *et al.* (2009) [14] also found chlortetracycline in swine, chicken, and cow fecal samples (1,150 µg/kg, 1,090 µg/kg and 2,220 µg/kg, respectively). A recent study also showed that TCs were detected in the range < 10 - 1,380 µg/kg and chlortetracycline was commonly found at high concentrations (46 - 404,000 µg/kg) in pig slurry [15]. Ofloxacin was detected in every sample. In chicken fecal samples, ofloxacin was found with a mean concentration of 1.516 µg/kg faeces lower than swine and cow faeces (9,280 µg/kg and 7,405 µg/kg, respectively). Norfloxacin was only found in cow fecal samples (2,479.67 µg/kg) whereas it was not detected in the other two types. Li Yan-Xia *et al.* (2012) [13] also found that flouroquinolones concentrations ranged from 380 µg/kg to 4,460 µg/kg in every sample, of which norfloxacin mean concentration was 2,280 µg/kg in all fecal samples. Tylosin mean concentrations were determined in chicken and cow fecal samples to be higher than 798.67 µg/kg and 5,802 µg/kg, respectively.

This study clearly demonstrates that raw faeces often contains antimicrobial residues. Most fecal samples are randomly taken from the breeding farms. This also implies that this study is not suitable for drawing conclusions about the stability of the antimicrobial residues in the faeces. Despite many efforts to reduce the use of veterinary antimicrobials, it is remarkable that nearly all fecal samples contained antimicrobial residues. The farmers shared data about antimicrobial usage in the months before the sampling. We have selected seven antimicrobials for this study. In some fecal samples, we were able to detect the presence of antimicrobials. For example, norfloxacin was not found in chicken and swine fecal samples, ormethoprim and tetracycline were not detected in cow and swine fecal samples. Although we are not able to detect these antimicrobials, we are not certain if this is due to the degradation of these antimicrobials or that these antimicrobials are not used. The fast degradation of these antimicrobials does not mean that they have no impact on the resistance, as they can exert pressure on the microbiota in the animal itself during treatment.

4. CONCLUSIONS

As shown above, antimicrobial levels in chicken, cow, and swine fecal samples are quite different. The total antimicrobial used a lot in chicken, cow, and swine husbandry was 60,980

µg/kg, 52,334 µg/kg, and 32,004.667 µg/kg, respectively. The predominant antimicrobials in cow breeding are diaminopyrimidines (trimethoprim), tetracyclines (chlortetracycline), flouroquinolones (ofloxacin and norfloxacin), and macrolides (tylosin); the predominant group in swine farming are diaminopyrimidines (trimethoprim), flouroquinolones (ofloxacin), and tetracyclines (chlortetracycline). Diaminopyrimidines (trimethoprim) and tetracyclines (tetracycline and chlortetracycline) in chicken husbandry are more than in swine and cow breeding.

In summary, we investigated the types of antimicrobials used in feeds and treatment aimed at Vietnamese chicken, cow, and swine production, and from these data we analyzed antimicrobials through their faeces. The results obtained clearly indicate that chicken production uses higher amounts of antimicrobials compared with cow and swine production. These data are intended to stimulate discussion as well as provide a baseline for further studies on antimicrobial use in husbandry. It would also be desirable to monitor the sales of antimicrobials to the veterinary drug industry and feed mills in Vietnam. These changes should be introduced along with guidelines for improving disease control by other means such as better hygiene and biosecurity on farms such as vaccination, or the addition of probiotics, prebiotics, or bioactive compounds in fodder can be used to protect vast herds from infection.

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CRediT authorship contribution statement. Le Thi Bach Tuyet: Conceptualization, Writing - Original Draft, Data Analysis. Do Trong Nghia: Editing, Supervision.

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.

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