UNIVERSIDAD DE CHILE
Campus Sur

DOCTORADO EN CIENCIAS SILVOAGROPECUARIAS Y VETERINARIAS

EVALUACIÓN DE LA DISEMINACIÓN DE RESIDUOS DE OXITETRACICLINA DESDE DEYECCIONES DE POLLOS BROILER TRATADOS HACIA CAMA Y AVES NO TRATADAS Y SU EFECTO SOBRE LA SELECCIÓN DE BACTERIAS RESISTENTES

Ekaterina Pokrant Huerta

Tesis para optar al Grado de Doctor en Ciencias Silvoagropecuarias y Veterinarias

PROFESOR GUÍA: JAVIERA CORNEJO KELLY
PROFESOR CO-GUÍA: LISETTE LAPIERRE

SANTIAGO, CHILE
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Facultad de Ciencias Agronómicas

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RESUMEN

La Oxitetraciclina (OTC) es un antimicrobiano utilizado en la industria avícola, a través del alimento y agua de bebida, sin embargo, se absorbe pobremente en el tracto gastrointestinal, por lo que es excretado por las aves. Es así como persiste en las deyecciones, principal componente de la cama de broiler. Con el objetivo de determinar la eliminación y diseminación de OTC hacia la cama y otros animales, y su efecto sobre la selección de cepas de *Escherichia coli* resistentes durante el post-tratamiento, se cuantificaron residuos de OTC+4-epi-OTC en deyecciones y cama de aves tratadas y no tratadas (centinelas), mediante un método validado por LC-MS/MS. Desde todas las muestras se aisló *E. coli* y se determinó su sensibilidad a tetraciclinas, mediante el método Kirby-Bauer. A partir de muestras obtenidas el día uno post-tratamiento, 50 aislados resistentes fueron tipificados mediante PFGE y se analizó la presencia de genes *tet* mediante PCR. Concentraciones promedio de OTC+4-epi-OTC entre 347,63-2244,66 µg/kg, se detectaron en el grupo tratamiento, y en los grupos centinela los residuos estaban bajo el límite de detección (12,5 µg/kg). La mayor proporción de cepas de *Escherichia coli* resistentes a tetraciclinas, fueron aisladas de las aves tratadas y se determinó que la susceptibilidad de las cepas es dependiente del grupo. Se observó una gran diversidad clonal de los aislados y el 80% presentó el gen *tet*(A). Se concluye que residuos de OTC+4-epi-OTC se eliminan y pueden ser traspasados desde aves tratadas hacia aves no tratadas, en donde trazas de este antimicrobiano fue detectado.

Palabras Claves: Oxitetraciclina; deyecciones; cama de broiler; *Escherichia coli,* genes *tet*.

ABSTRACT

Oxytetracycline (OTC) is an antimicrobial used in the poultry industry through feed and drinking water. However, it is poorly absorbed in the gastrointestinal tract, so it is excreted by birds. So, these residues persist in droppings, which it is the principal component of the broiler litter. In order to determine the elimination and dissemination of OTC to litter and other animals, and its effect on the selection of resistant *Escherichia coli* strains during post-treatment, OTC+4-epi-OTC residues were quantified in droppings and litter from treated and untreated birds (sentinel), by a validated LC-MS/MS method. *Escherichia coli* was isolated from all samples and its susceptibility to tetracyclines was determined by the Kirby-Bauer test. Fifty resistant isolates, from one post-treatment day samples, were examined by PFGE, and for the presence of *tet* genes by PCR. Average OTC+4-epi-OTC concentrations between 347.63 and 2244.66 µg/kg were detected in the treatment group. On the other hand, in the sentinel groups the residues were below the detection limit (12.2 µg/kg). The highest proportion of tetracycline-resistant *Escherichia coli* strains were isolated from the treated birds and the susceptibility of the strains was found to be group-dependent. A high clonal diversity of the isolates was observed and 80% presented the *tet*(A) gene. It is concluded that residues of OTC+4-epi-OTC are excreted and can be transferred from treated to untreated birds, where traces of this antimicrobial were detected.

Palabras Claves: Oxytetracycline; droppings; broiler litter; *Escherichia coli, tet* genes.

INTRODUCCIÓN

La industria avícola se ha caracterizado por ser una de las áreas del sector pecuario con mayor crecimiento, debido principalmente a que la carne de ave se ha transformado en una fuente de proteína animal económicamente asequible. Según las estimaciones realizadas por la *Food and Agriculture Organization* (FAO), de los 328 millones de toneladas de carne mundial producida en el año 2020, la carne de ave correspondió a un 36,5%, equivalente a 134 millones de toneladas, un 1,2% más que en el año 2019. Para el año 2030 se proyecta una producción de un 60% más de carne de ave (OECD/FAO, 2021).

Este aumento de la producción avícola, para satisfacer la creciente demanda mundial de proteína animal, conlleva a una de las principales preocupaciones para la práctica veterinaria, que es garantizar la sanidad animal, por lo que los antimicrobianos siguen siendo una herramienta fundamental para el tratamiento de enfermedades infecciosas de origen bacteriano (Fukuda, 2015; Weese *et al*., 2015; NRC 2017).

No obstante, el uso terapéutico de estos fármacos en animales de producción no está exento de riesgos para la salud animal y pública, debido a que se ha descrito que a través de las deyecciones de pollos de engorde se excretan residuos de antimicrobianos, los cuales persisten por un periodo más prolongados que el periodo de resguardo establecido para las formulaciones farmacéuticas utilizadas, y en mayores concentraciones que los límites máximos residuales (LMR) establecidos para los tejidos comestibles (Berendsen *et al*., 2013; Cornejo *et al*., 2018; Yévenes *et al.,* 2018).

Los LMR fueron establecidos en productos alimenticios y de origen animal, por diferentes organismos gubernamentales para proteger a los consumidores de la exposición a los residuos antimicrobianos (Berendsen *et al.,* 2011), considerando el riesgo que representan estos medicamentos para la salud pública (CE, 2009; *Codex Alimentarius* 2017). Una vez establecidos los LMR, se puede monitorear y controlar la presencia de residuos de antimicrobianos en los alimentos. Sin embargo, en el caso de los subproductos, al no ser destinados para consumo humano directo, no existen LMR establecidos, no existiendo un monitoreo ni control de residuos.

La importancia de esto radica en que estos subproductos no comestibles, como la cama de broiler, compuesta principalmente por deyecciones, es utilizada en sistemas agrícolas como fertilizante y como suplemento alimenticio para otras especies productivas, ya que es buena opción alimenticia, sobre todo para rumiantes (Gadberry, 2014). En nuestro país, la

Resolución Exenta N°7352 del 25 de noviembre del 2010 aprueba el Instructivo Planteles de Animales Bovinos Bajo Certificación Oficial (PABCO Bovinos), en la cual se prohíbe a aquellos planteles bovinos PABCO nivel “A” la alimentación con estiércol o guano de cualquier especie (SAG, 2010). No obstante, un gran número de productores a nivel nacional presenta la certificación PABCO nivel “B”, esta certificación permite la utilización de estiércol o guano, como cama de broiler, en la ración alimenticia de los animales (Álvarez, 2009).

Considerando que los residuos de estos fármacos se excretan como metabolitos activos o generalmente en su forma original en concentraciones de mg/kg o μg/kg (Li *et al.,* 2013; Hou et al., 2015; Berendsen *et al*., 2018), la cama de broiler podrían ser un riesgo para los consumidores, al ser una posible ruta de reingreso de residuos de antimicrobianos a través de la cadena alimentaria y de transferencia a otros animales que no hayan sido tratados con antimicrobianos (Stahl *et al.,* 2016).

La principal preocupación por la excreción y persistencia de estos medicamentos en la cama de broiler, es sobre su contribución a la resistencia antimicrobiana (Beyene 2016; Van Boeckel *et al*., 2017). Se ha descrito que la presencia de residuos de antimicrobianos en el medio ambiente contribuye significativamente a la selección de bacterias resistentes a antimicrobianos (Fletcher, 2015; Manyi-Loh *et al.,* 2018). Considerando que las bacterias fecales pueden sobrevivir durante semanas o meses en el medio ambiente (Chee-Sanford *et al.*, 2009), el estudio en esta área ha cobrado relevancia. Durante la última década ha aumentado la información científica disponible sobre el riesgo para la salud pública debido a la selección de bacterias resistentes en animales productores de alimentos (CIPARS, 2015; EFSA, 2017).

Las terapias con antimicrobianos pueden afectar tanto a las bacterias patógenas a las cuales están dirigidas, como a bacterias comensales, alterando de esta manera la microbiota normal. El impacto sobre la población comensal depende del antimicrobiano utilizado, su mecanismo de acción y el nivel de resistencia de la población bacteriana (Jernberg *et al*., 2010). Estudios realizados en animales y humanos, han mostrado que la administración de antimicrobianos, incluso por cortos periodos de tiempo, puede permitir la estabilización de poblaciones bacterianas intestinales resistentes durante años (Faldynova *et al*., 2013).

Por su parte, los elementos genéticos móviles y genes de resistencia pueden persistir independientemente de la viabilidad celular (Chee-Sanford *et al*., 2009). Estos últimos, se han descrito como un contaminante ambiental emergente, debido a su potencial de afectar negativamente la salud humana y animal (Sanderson *et al.,* 2016). Por consiguiente, la

transmisión de cepas resistentes y genes de resistencia entre los animales y los seres humanos en el camino de la granja al tenedor es hoy en día una gran preocupación en la salud animal y humana (Founou *et al*., 2016). Por lo tanto, las dos áreas que abarca esta tesis doctoral son a) calidad e inocuidad alimentaria y b) biomedicina y prevención de enfermedades.

A pesar de los grandes esfuerzos realizados por organizaciones internacionales como la FAO, la OIE y la OMS para abordar los principales vacíos de información y comprender la gravedad del problema, hasta ahora no se ha abordado el papel de los sistemas productivos y el manejo de desechos animales (Fletcher, 2015). Es por este motivo, que la generación de información científica respecto a esta temática es de suma importancia.

Considerando lo previamente expuesto, y que la oxitetraciclina es un antimicrobiano perteneciente a la familia de las tetraciclinas y ampliamente utilizado en producción avícola, es que el estudio del comportamiento y la dinámica de diseminación de este antimicrobiano cobra relevancia. En conjunto a esto, y considerando la importancia de las tetraciclinas sobre la resistencia a los antimicrobianos, es apropiado determinar el efecto que pueda tener la persistencia de residuos de este antimicrobiano en la selección de bacterias resistentes en uno de los principales subproductos de la industria avícola.

HIPÓTESIS

“Los residuos de Oxitetraciclina (OTC) eliminados desde deyecciones de pollos broiler tratados se diseminan hacia el ambiente (cama y aves no tratadas), persistiendo en el tiempo y favoreciendo la selección de bacterias resistentes en estos compartimentos”.

Objetivo general

Evaluar la propagación de residuos de OTC desde deyecciones de pollos broiler tratados hacia la cama y aves no tratadas, su persistencia durante el periodo postratamiento y el efecto de estos residuos sobre la selección de cepas bacterianas resistentes de *E. coli*, como bacteria indicadora, en las matrices de interés.

Objetivos específicos

Objetivo 1: Implementar y validar métodos analíticos para la detección y cuantificación de OTC y 4-epi-OTC, desde las matrices de estudio (deyecciones y cama de broiler).
Objetivo 2: Evaluar la diseminación de residuos de OTC y 4-epi-OTC desde deyecciones de pollos broiler tratados hacia diferentes áreas: cama y aves no tratadas durante el periodo postratamiento.
Objetivo 3: Determinar la relación entre las concentraciones de residuos de OTC y 4-epi-OTC con la presencia de cepas bacterianas no susceptibles a este antibiótico y la presencia de determinantes de resistencia en estas bacterias, en las diferentes matrices analizadas (deyecciones de aves tratadas, aves no tratadas y sus respectivas camas).

ÍNDICE DE CAPÍTULOS

CAPÍTULO 1: *"Evaluation of Antibiotic Dissemination into the Environment and Untreated Animals, by Analysis of Oxytetracycline in Poultry Droppings and Litter".*
Animals 2021, 11(3):853. https://doi.org/10.3390/ani11030853

Este capítulo aborda los objetivos específicos 1 y 2.

CAPÍTULO 2: *"Evaluación de la resistencia fenotípica y genotípica de cepas de Escherichia coli aisladas desde deyecciones y cama de pollos broiler posterior al tratamiento con oxitetraciclina".*

Este capítulo aborda el objetivo específico 3.

CAPÍTULO SUPLEMENTARIO: *"Determination of five antimicrobial families in droppings of therapeutically treated broiler chicken by high-performance liquid chromatography-tandem mass spectrometry".*
Poultry Science 2021, 100:101313. https://doi.org/10.1016/j.psj.2021.101313

Este capítulo aborda parcialmente el objetivo 1 y 2.

CAPÍTULO 1

Article

Evaluation of Antibiotic Dissemination into the Environment and Untreated Animals, by Analysis of Oxytetracycline in Poultry Droppings and Litter

Ekaterina Pokrant [1,2,*], Karina Yévenes [1,2], Lina Trincado [1,2], Gigliola Terraza [1], Nicolás Galarce [1], Aldo Maddaleno [2,3], Betty San Martín [3], Lisette Lapierre[1] and Javiera Cornejo [1,*]

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Simple Summary: Oxytetracycline (OTC) is an antibiotic used mainly in feed and drinking water. OTC is poorly absorbed in the gastrointestinal tract of poultry; making droppings a potential route of dissemination of this antibiotic. The aim of this study was to evaluate the dissemination of oxytetracycline excreted from treated birds to the environment and other untreated animals (sentinels), through the analysis of their droppings and litter by HPLC-MS/MS following the end of treatment. In treated bird droppings, the average concentration of OTC+4-epi-OTC ranged from 347.63 to 2244.66 μg kg^{-1}. OTC+4-epi-OTC in litter reached concentrations of 22,741.68 μg kg^{-1}. Traces of OTC+4-epi-OTC were detected in the droppings and litter from sentinels. Therefore, OTC+4-epi-OTC can persist in the litter of treated animals at high concentrations and can be transferred to untreated birds that share the same environment. This exposure has the potential to increase the likelihood of selection of resistant bacteria in the environment.

Abstract: Oxytetracycline (OTC) is widely used in broiler chickens. During and after treatment a fraction of OTC is excreted in its original form and as its epimer, 4-epi-OTC in droppings. To address the transfer of OTC into the environment, we evaluated the dissemination of OTC and 4-epi-OTC from treated birds to the environment and sentinels, through the simultaneous analysis of broiler droppings and litter. Male broiler chickens were bred in controlled conditions. One group was treated by orogastric tube with 80 mg kg^{-1} of OTC and two groups received no treatment (sentinels). OTC+4-epi-OTC were analyzed and detected by a HPLC-MS/MS post the end of treatment. The highest concentrations of OTC+4-epi-OTC were detected in the droppings of treated birds 14-days following the end

of treatment (2244.66 μg kg^{-1}), and one day following the end of treatment in the litter (22,741.68 μg kg^{-1}). Traces of OTC+4-epi-OTC were detected in the sentinels' droppings and litter (<12.2 μg kg^{-1}). OTC+4-epi-OTC can be transferred from treated birds to the environment and to other untreated birds. The presence and persistence of OTC+4-epi-OTC in litter could contribute to the selection of resistant bacteria in the environment, increasing the potential hazard to public and animal health.

Keywords: Oxytetracycline; 4-epi-Oxytetracycline; poultry droppings; poultry litter; broiler chicken; HPLC-MS/MS; antibiotic dissemination; sentinel birds.

1. Introduction

Tetracyclines, which were discovered in the 1940s, are broad spectrum bacteriostatic antibiotics [1,2]. In some countries, such as Brazil and China these antibiotics can be used to promote growth in farm animals. However, this practice was banned in Europe in 2006 and in the USA in 2017 [3,4]. The third Annual Report of the World Organization for Animal Health (OIE) on the Use of Antimicrobial Agents in Animals, states that tetracyclines were the most used class of antimicrobial in 166 countries between 2015 and 2017 [5]. Currently, more than 20 tetracyclines are available; tetracycline, chlortetracycline, oxytetracycline, and doxycycline are the most used in veterinary medicine [3].

Oxytetracycline (OTC) is used in the poultry industry and is commonly administered to chickens through feed and drinking water to treat several diseases, such as chronic respiratory disease, infectious coryza, and fowl cholera [1,6,7]. When OTC is administrated to birds either for treatment or as growth promoter, residues of the antibiotic persist in their products and by-products, and are excreted in droppings [6,8–11]. Analyses of the OTC pharmacokinetics in broiler chickens suggests that OTC is poorly absorbed from the gastrointestinal tract of birds, and is therefore excreted in droppings at a higher concentration when administered orally [12,13]. Other studies have found antimicrobials in the feces of animals, such as swine and bovine. Oxytetracycline, doxycycline and sulfadiazine were most frequently detected, followed by tetracycline and other antimicrobials. Animals can excrete a significant proportion of the administered antibiotics (17–90%) unchanged or as active metabolites (epimers or isomers) of the parent antimicrobial [14].

Weakly acidic conditions favor the transformation of OTC to the 4-epi-oxytetracycline (4-epi-OTC) epimer, which has approximately 30% of the antibacterial activity of its precursor [15–17]. Wang et al. [16] showed that the peak concentration of 4-epi-OTC detected in the manure of treated pigs was 1337.08 mg kg^{-1} and that this metabolite influenced OTC degradation. Therefore, the European Union (EU) considers the sum of both OTC and 4-epi-OTC, as a marker to monitor OTC residues in animal products [18]. However, antibiotic residues are not monitored in non-edible by-products, such as chicken droppings which form an important part of broiler litter, along with feather remains, shavings and feed scraps [19].

Various studies have detected OTC in poultry droppings, manure, and litter. Zhao et al. [20] detected OTC in the manure of chickens, with a relatively high occurrence of 27.8% of sampled birds and a geometric mean of 1.55 mg kg^{-1}. Similarly, Li et al. [21] confirmed the presence of OTC in dropping samples from 18 chicken farms in the northeastern provinces of China. Tetracyclines were the most prevalent of all antimicrobial families tested for in the study and the occurrence of OTC in chicken droppings was 44.4% of samples with a range of 0.54 to 4.57 mg kg^{-1} [21]. Further research in Iran has identified the concentration of OTC in broiler manure from 25 different farms, where the levels ranged from 0.047 to 13.77 mg kg^{-1} [22]. In this context, in Egypt, Mahmoud and Abdel-Mohsein [23], found OTC concentrations from 5.9–1.33 μg g^{-1} in poultry litter and droppings.

In vitro analyses of OTC residues in manure suggest that the half-life is 8.1 days in 80% humidity [24]. Berendsen et al. [25] determined that OTC has a longer half-life than chlortetracycline (CTC) in stored broiler manure. The presence and persistence of OTC in droppings and poultry litter is relevant because the by-products are used in the feed of other production animals and are re-applied to land as fertilizer [14,26]. Also, poultry litter can contribute to the dissemination of antibiotics to the environment and other untreated animals through dust which can carry antibiotic residues [27]. The carryover of antibiotic residues to untreated animals through the environment has been experimentally demonstrated by Stahl et al. [28], where traces of sulfadiazine were detected in plasma and urine of untreated pigs kept in a barn that was previously used to treat pigs. The dissemination of OTC has not been fully elucidated and the presence and persistence of this antibiotic in droppings and litter represents a hazard to the environment, as well as to public and animal health. Therefore, it is important to determine the concentrations of OTC that persist in these animal waste.

Tetracyclines are used world-wide in animal production, increasing the likelihood for antimicrobial resistance. Thus, surveillance of these antibiotics is paramount [3]. Consequently, the aim of this study was to evaluate concentrations of OTC and 4-epi-OTC in the droppings and litter of therapeutically treated birds and also of untreated birds, kept in nearby pens, in order to assess antimicrobial environmental dissemination. For this purpose, an analytical methodology by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was optimized and validated to ensure the reliability and precision of the results obtained in droppings and litter samples of treated and untreated animals.

2. Materials and Methods

2.1. Experimental Animals

Male broilers from the Ross 308 genetic line (Ross®, Aviagen Inc., Huntsville, AL, USA) were raised from birth in an experimental unit specially designed to carry out this study. In this experiment, 1.5 m^2 corrals were conditioned with clean shavings that later became part of the birds' litter. Environmental conditions such as temperature (25 ± 5 °C), humidity (50–60%) and ventilation were controlled.

During the experiment, the birds had ad libitum access to water and non-medicated feed, which was previously analyzed by HPLC-MS/MS to verify the absence of OTC residues. The diets were formulated according to the nutritional requirements of the breed as recommended in the Aviagen™ manual [29].

The protocol for the management and monitoring of experimental birds was based on Law No. 20.380 "On the Protection of Animals" [30] and Directive 2010/63/EU on the protection of animals used for scientific purposes [31]. Regulation (EC) No 1099/2009 on the protection of animals at the time of killing [32] was respected. The Institutional Committee for the Comité Institucional de Cuidado y Uso de Animales (CICUA) of the University of Chile approved the use of the birds for the experimental study, certificate N°: 18187-VET-UCH-E1.

2.1.1. Animal Groups and Treatment

In this study, the birds were randomly assigned from the first day of hatch to three experimental groups. Group A included 6 birds treated with a pharmaceutical formulation containing OTC at 100 mg mL^{-1} (10%), which is authorized for use in fattening birds. All birds in group A were treated orally using an orogastric tube (Levin No. 6) and a sterile syringe, with a therapeutic dose of 80 mg kg^{-1} for 10 consecutive days. The drug was administered directly into the crop, ensuring the delivery of calculated doses. The birds were weighed daily, and the dosage calculated according to individual weight was administered. (Tables S1 and S2). The second and third group (Groups B and C) also contained 6 birds each and were untreated (sentinel birds). To determine the transfer of OTC residues from treated birds to untreated birds group B was kept in an adjacent pen to group A while group C was kept 30 cm from group A (Figure 1). In this latter, an effective separation of 30 cm was determined in order to avoid the total direct contact between the birds. All pens had an area of 1.5 m^2, divided by a solid wall of 1.5 m in height. All measures were taken to avoid contamination by handling, such as the use of shoe covers, gloves, and protective implements for the handling of each experimental group.

Figure 1. Schematic diagram of the placement of experimental groups; on the left group C, sentinels 30 cm from treated birds; in the center group A, birds treated with 80 mg kg^{-1} of OTC; and on the right group B, sentinels in adjacent pens.

2.1.2. Sampling Collection

Six samples of droppings and litter were collected per sampling point, corresponding at days 1, 7, 14, and 21 post the end of treatment. Litter samples were also collected on days 1, 7, and 14 post-slaughter (up to 22 days post the end of treatment), to determine the persistence of OTC in the pens without experimental birds. The day of slaughter was at 42 days of hatch, corresponding to the slaughter day of broilers in the industry (Figure 2).

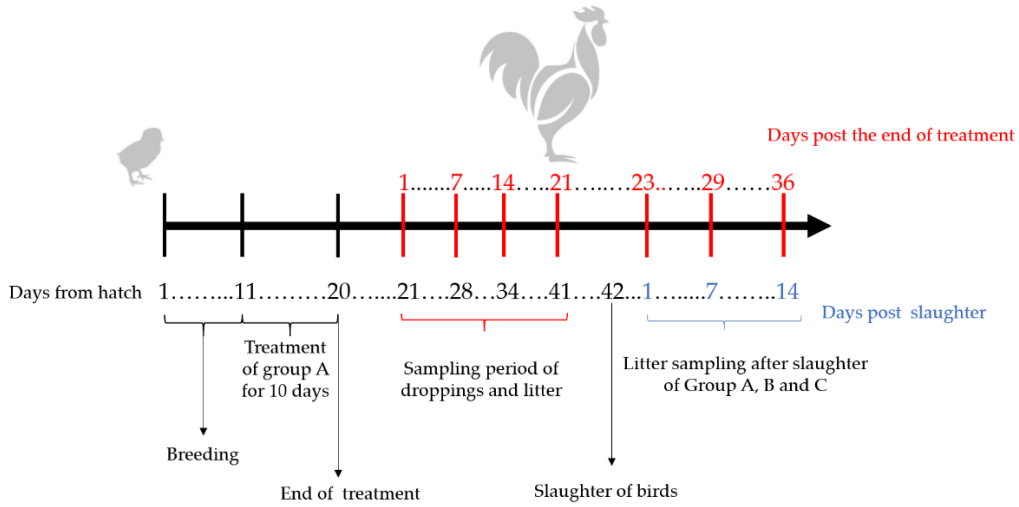


Figure 2. Schematic diagram of treatment and sampling period.

Five grams of dropping were obtained from the cloaca of each bird by stimulation with sterile torulas and were stored individually in sterile polypropylene tubes at −20 °C until chemical analysis. Meanwhile, ten grams of litter samples were collected from Group A, B and C. Six samples were obtained at each time point in accordance with the Servicio Agrícola y Ganadero (SAG) soil sampling protocol, which establishes the requirements to comply with the parameters to be evaluated under Article 28 of the DS: No. 4/09 [33]. According to this. samples were collected equidistantly within each pen, covering the whole area, using an asystematic sampling method, as suggested by the protocol mentioned above. Litter samples were stored at −20 °C, in properly labelled plastic bags until further processing and analysis.

2.2. Chemical Analysis

2.2.1. Reagents, Solvents and Standards

For sample fortification, certified standards of OTC and 4-epi-OTC were purchased from Dr. Ehrenstorfer™. Tetracycline-d6 (TC-d6) was used as an internal standard (IS) and was purchased from Toronto Research Chemicals (Toronto, Canada). A stock solution of OTC, 4-epi-

OTC and TC-d6 was prepared in methanol at a concentration of 1000 µg mL^{-1} and stored at −80 °C. Intermedia or working solutions were prepared using a diluted stock solution at a concentration of 1000 ng mL^{-1} and stored at −80 °C.

Acetonitrile, methanol, and water were used for extraction of analytes from matrices. All solvents were from LiChrosolv® (MERCK KGaA, Darmstadt, Germany) line and LC-MS grade. Buffer EDTA-McIlvaine was also used for the extraction procedure. It was prepared from 0.1 M citric acid and 0.2 M disodic phosphate. The McIlvaine buffer was prepared by mixing 500 mL of 0.1 M citric acid solution with 280 mL of 0.2 M disodium phosphate. The solution was brought to 2 liters with Milli-Q® water and the pH adjusted to 4.0 ± 0.1 with 0.1 M citric acid or 0.2 M disodic phosphate. For the 0.1 M Na2EDTA-McIlvaine buffer, 74.4 g of Na_2EDTA (Titriplex® III ACS, ISO, Reag. Ph Eur (MERCK KGaA, Darmstadt, Germany) was dissolved with the EDTA-McIlvaine buffer previously prepared.

For chromatography analysis, 0.1% formic acid in water with a pH 2.7 ± 0.2 (eluent A) and 0.1% formic acid in methanol with a pH 3.0 ± 0.3 (eluent B) were used.

2.2.2. Extraction Procedure

The extraction procedure was based on the previously published protocol of Berendsen et al. [13]. The protocol was optimized and validated for the detection of OTC and 4-epi-OTC in droppings and litter. For the analysis, 1 ± 0.01 g of sample was homogenized and weighed in a 50 mL polypropylene tube, 8 mL of EDTA-McIlvaine buffer solution and 2 mL of acetonitrile were used for extraction of OTC+4-epi-OTC. Samples were filtered through Whatman™ filter paper grade GF/A (1.6 µm) (MERCK) and then the extract was applied to a solid phase extraction column (SPE) Supel™ Select HLB (Supelco, MERCK KGaA, Darmstadt, Germany), previously conditioned with 5 mL of methanol and 5 mL of water LC-MS grade. The columns were then washed with 5 mL of water LC-MS grade, dried with a manifold pump for 5 min and finally eluted with 10 mL of methanol LC-MS grade. The samples were dried under a flow of mild nitrogen in a water bath between 40–50 °C in an automated solvent evaporation system (TurboVap® LV, Biotage, Uppsala, Sweden) and reconstituted with 200 µL methanol and 300 µL water HPLC grade, and finally transferred to glass vials for chromatographic analysis by HPLC-MS/MS.

2.2.3. Instrumental Analysis

All samples were analyzed using a high-performance liquid chromatography system, consisting of an Agilent 1290 Infinity autosampler and thermostat and Agilent 1260 Binary pump, coupled to a triple quadrupole tandem mass spectrometer, in multiple reaction monitoring mode (MRM) through an electrospray interface. An API 5500 (AB Sciex, Darmstadt, Germany) mass spectrometer was used. This device was operated in positive ionization mode for the detection of OTC, 4-epi-OTC, and TC-d6 analytes.

The samples were processed and analyzed at the Laboratory of Veterinary Pharmacology (FARMAVET) of Faculty of Veterinary and Animal Sciences, of the University of Chile. This laboratory works under required biosecurity measures, good laboratory practices, and is accredited under ISO 17025 standards.

2.3. Validation of Analytical Methodology

Analytical methods are an essential tool for performing drug residue evaluation in edible and non-edible matrices. In order to ensure that the method is able to provide reliable and accurate data previous in-house laboratory validation is needed [34]. For this aim, validation of the method was performed through different procedures to ensure that the method detects and quantify OTC and 4-epi-OTC in droppings and litter by HPLC-MS-MS, precisely and confidently.

The analytical method was validated following an internal protocol based on the recommendations from the regulation and guidance of Commission Decision 2002/657/EC [35] and VICH GL49 [36]. The parameters evaluated were retention time, limit of detection (LOD) and limit of quantification (LOQ), specificity, recovery, linearity of the calibration curve and precision (by repeatability and intra-laboratory reproducibility). LOD and LOQ of the analytical methodology, linearity of the calibration curve and method recovery, were considered as critical parameters for accurately quantify OTC residues in experimental samples. All parameters and acceptance criteria are described in Supplementary Table S4.

3. Results

3.1. Optimization and Validation of Analytical Method

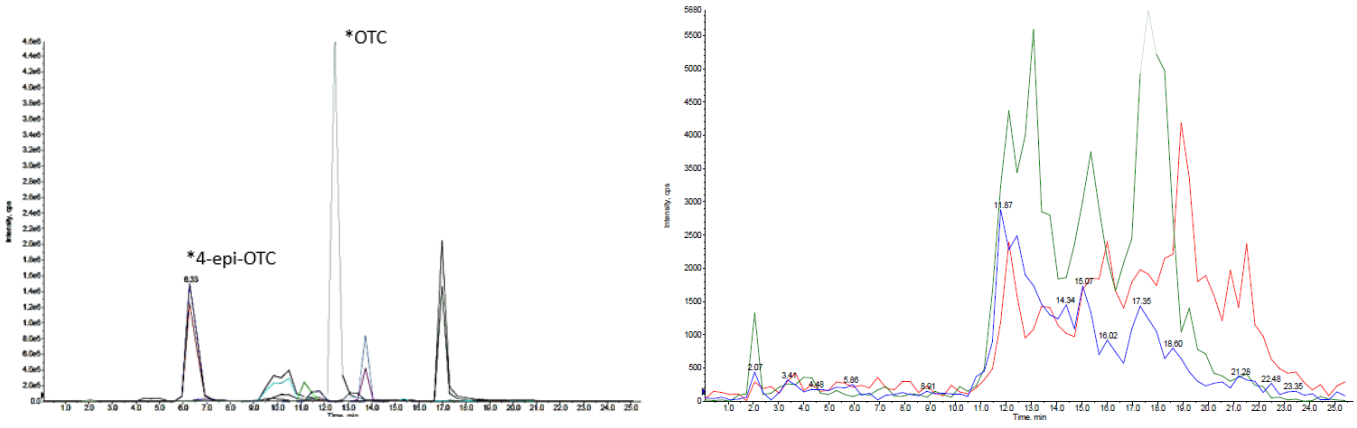
During the implementation, the sample volume was decreased, and the volume of extraction solvents was increased. In addition, filtering the samples through Whatman™ microfiber glass paper filters, grade GF/A (1.6 μm) (MERCK) was added. These last two aspects were modified to improve the clean-up of the samples, since the droppings and poultry litter are highly complex matrices with many interferents that affect the reading and chromatographic analysis.

During the optimization, the optimal condition for chromatographic analysis were set. The reversed-phase column Sunfire ™ C 18 3.5 μm, 2.1 mm × 150 mm (Waters Corp, Milford, MA, USA) was used for the analytical separation, with a temperature of 35 ± 1 °C, the injection volume corresponded to 20 μl, with a flow rate of 0.2 mL min^{-1}. Eluent A was 0.1% formic acid in water with a pH 2.7 ± 0.2 and eluent B was 0.1% formic acid in methanol with a pH 3.0 ± 0.3. The analysis in the mass spectrometer was trough electrospray ionization (ESI), with a source temperature of 550 °C, and curtain and collision gas of 20 and 10 psi, respectively. The positive mode ion spray voltage was set at 45,000 V, and the ion source gas 1 and 2 at 60 and 80 psi, respectively. Table S3 shows the precursor and product ions for each analyte and the programming of the mass spectrometer to capture the respective ion products.

All parameters, according to the internal validation protocol, fulfilled the criteria following guidelines 2002/657/EC and VICH GL49 [35,36]

(Table S4). The average retention time for OTC (461.0/426.0 Da) was 11.632 ± 0.0571 min, and for 4-epi-OTC (461.0/426.0 Da) the average retention time was of 6.913 ± 0.0423 min (Figure 3).

The method is specific for the detection of OTC and 4-epi-OTC, according to the analysis of 20 free samples, per study matrix. No interference in the retention times of OTC and 4-epi-OTC was detected in any replicates. In Figure 3, a chromatogram representative of the injection of a certified standard of OTC and 4-epi-OTC, and a litter sample free of these analytes is shown, in the latter no interference in the retention times is observed.



(**a**) (**b**)

Figure 3. Representative chromatograms of the (**a**) injection of a certified standard of OTC and 4-epi-OTC (*: peaks) and from the analysis of (**b**) litter sample free of OTC and 4-epi-OTC.

The instrumental LOD and LOQ determined for OTC was 0.0027 and 0.0089 µg/g and 0.0023 and 0.0078 µg/g for 4-epi-OTC, respectively. The LOD and LOQ in droppings and broiler litter matrices are shown in table 1.

Table 1. Validation parameters for droppings and litter.

Matrix	Analyte	Linearity (R^2)[3] ± SD	Recovery[4] (%)	LD[5] (µg kg^{-1})	LC[6] (µg kg^{-1})
Droppings	OTC[1]	0.995 ± 0.004	104.3	12.128	36.384
	4-epi-OTC[2]	0.997 ± 0.003	98.4	12.157	36.470
Litter	OTC	0.993 ± 0.004	91.9	12.409	37.228
	4-epi-OTC	0.992 ± 0.007	96.0	10.783	32.349

[1] OTC: Oxytetracycline; [2] 4-epi-OTC: 4-epimer-Oxytetracycline; [3] Average R^2 (Coefficient of determination) ± Standard deviation of 3 calibration curves in fortified matrix at a concentration of 12.5, 25, 50 and 100 µg kg^{-1}, including zero; [4] Percentage of recovery from fortified matrix at a concentration of 25 µg kg^{-1}; [5] Limit of Detection in matrix; [6] Limit of Quantification in matrix.

The linearity for both analytes was determined by matrix curves fortified at concentrations of 12.5, 25, 50, and 100 µg kg^{-1} including zero,

all curves showed a coefficient of determination (R^2) greater than 0.99, in the two study matrices (Table 1).

The relative standard deviation (RSD) determined for precision, through repeatability and reproducibility within the laboratory, did not exceed the 23% of variation, during the analysis of fortified samples at the lowest concentration of 25 μg kg^{-1} for both matrices. The results of the repeatability and reproducibility tests are described in the supplementary material (Table S5). For all levels of fortification, the recovery percentages were within the acceptable ranges of ±10% (Table 1 and Supplementary Table S5).

3.2. Detection and Quantification of OTC and 4-epi-OTC in Experimental Samples

Prior to the start of the study, bird droppings and shavings used for the bird litter were sampled. No OTC residues were detected in any of the samples. OTC and 4-epi-OTC concentrations were detected in the droppings of treated birds throughout the post the end of treatment period. It was observed that the highest concentrations of OTC+4-epi-OTC were quantified on day 14 post the end of treatment (2244.66 μg kg^{-1}). OTC+4-epi-OTC concentrations were never below 300 μg kg^{-1} in treated birds (Table 2). Trace concentrations of OTC were detected groups B and C (sentinels). These levels were below the LOD in matrix (12.1 μg kg^{-1} for OTC and 12.2 μg kg^{-1} for 4-epi-OTC). An OTC+4-epi-OTC concentration of 9489.42 ug kg^{-1} was detected in only one dropping sample in Group C, 21 days after treatment (Table 2). For the analysis, the wet weight of the samples was considered. Droppings and litter samples had an average water content of 80% and 15%, respectively.

Table 2. OTC+4-epi-OTC concentrations in dropping and litter samples.

Days post the end of treatment (Days post-slaughter)	Average OTC+4-epi-OTC (μg kg^{-1}) in Droppings			Average OTC+4-epi-OTC (μg kg^{-1}) in Litter		
	Group A[1]	Group B[2]	Group C[3]	Group A[1]	Group B[2]	Group C[3]
1	2087.41	<LOD[4]	<LOD*	22,741.68	<LOD	<LOD
7	347.63	<LOD	<LOD*	15,594.05	<LOD	<LOD
14	2244.66	<LOD	N/D[5]	12,236.68	<LOD	<LOD
21	733.00	<LOD	N/D[5]	12,946.13	<LOD	<LOD**
23 (1)	-	-	-	10,360.60	<LOD	<LOD
29 (7)	-	-	-	15,557.62	<LOD	<LOD
36 (14)	-	-	-	11,429.14	<LOD	<LOD

[1] Treatment birds with 10% OTC; [2] untreated birds adjacent to treatment group; [3] untreated birds 30 cm from treated birds; [4] LOD: Limit of Detection (12.1 μg kg^{-1} for OTC and 12.2 μg kg^{-1} for 4-epi-OTC); [5] N/D: None Detected (no chromatographic signal of OTC and 4-epi-OTC above noise signal). * two samples show a response of OTC greater than 3 times the signal noise of the baseline. ** one sample measured 9489.42 μg kg^{-1} for OTC+4-epi-OTC.

In group A, concentrations of OTC+4-epi-OTC in litter samples exceeded concentrations in droppings by more than 10 times one day following the end of treatment. A representative OTC (461.0/426.0) chromatogram of a dropping and litter sample at 50 μg kg^{-1} and OTC

(461.0/426.0) chromatogram of experimental droppings and litter samples from the first sampling point of Group A are shown in Figure 4.

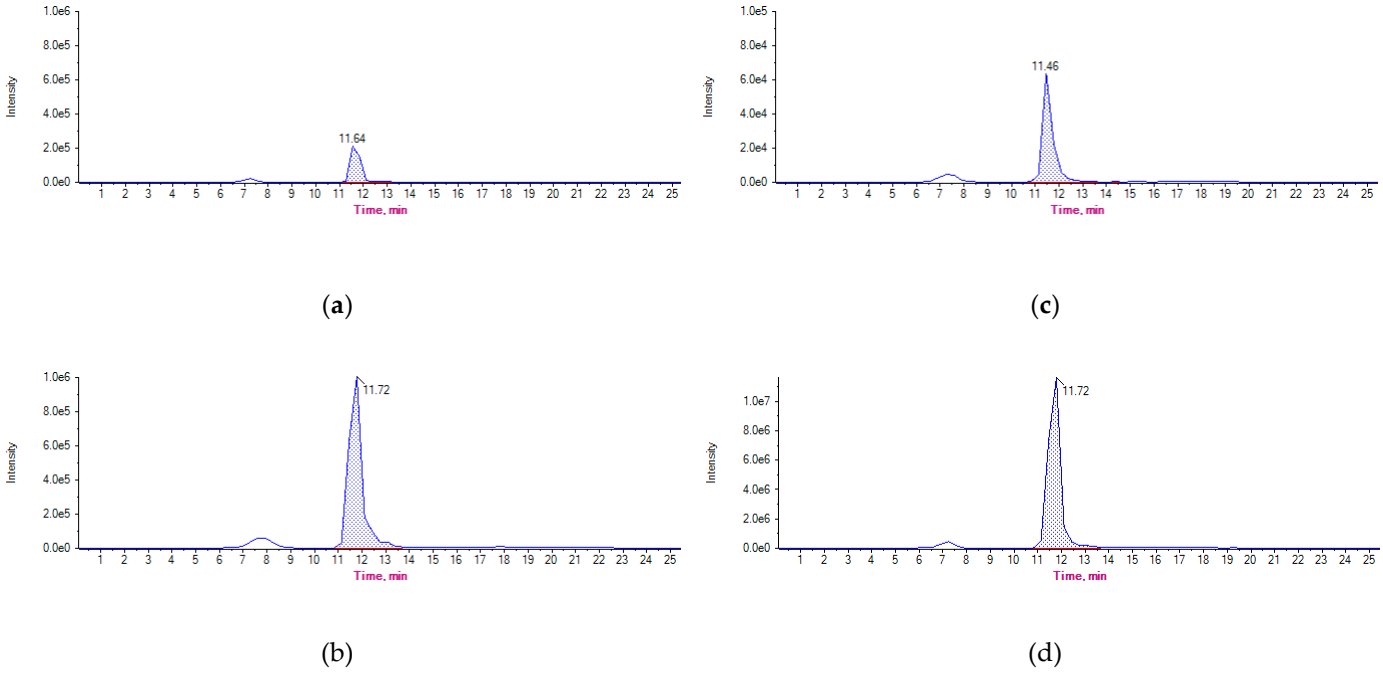
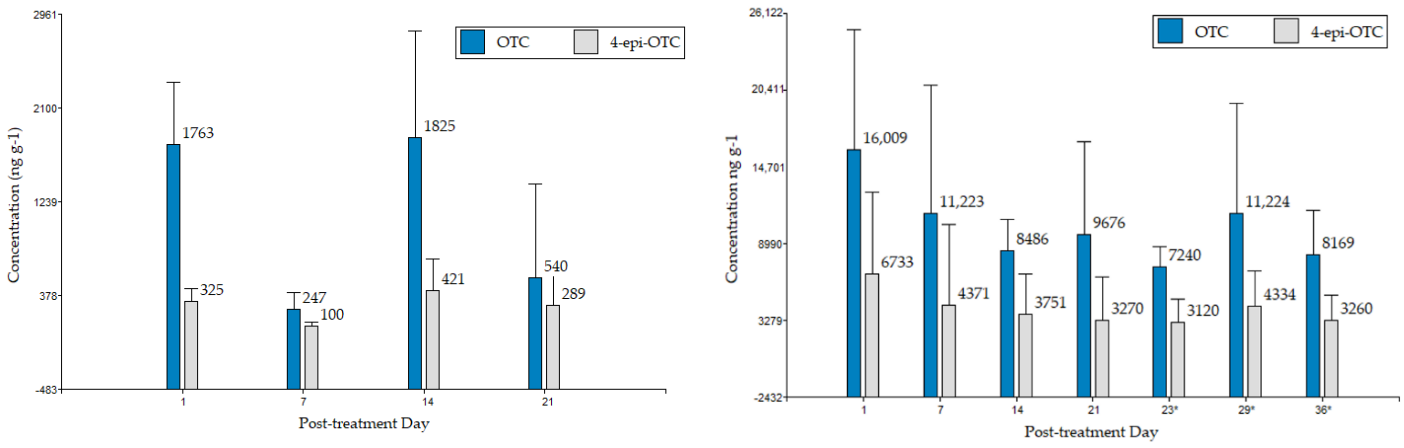


Figure 4. Chromatograms of OTC (461.0/261.0) from (**a**) droppings fortified at 50 μg kg^{-1}, (**b**) experimental droppings sample from group A day 1 post the end of treatment, (**c**) litter sample fortified at 50 μg kg^{-1} and (**d**) experimental litter sample from group A day 1 post the end of treatment.

In Figure 5, bar charts show the concentration of OTC and 4-epi-OTC separately, for group A. In litter OTC concentrations tend to decrease while the epimer is observed throughout. In droppings, concentrations varied throughout the course of sampling.

To determine differences between the concentration detected in droppings at days 1, 7, and 14 following the end of treatment, a t-test was performed. The detected concentrations were expressed as natural logarithm and the statistical software Infostat® [37] was used for the analysis. In all experiments, differences were considered statistically significant when the associated probability level (p) was less than 0.05.

The analysis shows that for the concentrations of both analytes detected at seven days following the end of treatment, were statistically different than those detected on days 1 and 14 following the end of treatment (p-value < 0.05). While the concentrations detected for both analytes on days 1 and 14 do not present significant differences.



(**a**) (**b**)

Figure 5. Concentrations of OTC and 4-epi-OTC in bird (**a**) droppings and (**b**) litter, from group A with 10% OTC, post the end of treatment and post slaughter (*: days post slaughter, correspond to 1-, 7- and 14-days post slaughter). Error bars represent the standard deviation.

4. Discussion

The analytical procedure for OTC and 4-epi-OTC detection by HPLC-MS/MS was optimized from work previously published by Berendsen et al. [13] with the aim of extracting OTC and its epimer, from droppings and broiler litter. The modifications made were to improve the cleaning of the samples, in order to reduce the presence of interfering substances that could interfere with the chromatographic analysis, since as previously described, manure is a complex matrix [38]. The optimized analytical method can detect and quantify accurately and reliably, concentrations of OTC and 4-epi-OTC from dropping and litter matrices. All parameters determined during the validation of the methodology met the acceptance criteria according to the recommendations of 657/2002/EC and VICH GL49 [35,36]. The linearity of method presented a R^2 greater than 0.99 for both sample types, although the dropping R^2 was higher than broiler litter. The difference may be due to the fact that litter is a heterogeneous sample composed of different structures, such as feather and feed remains [19], which makes the processing of the sample during the extraction procedure more complex. The validated method is accurate and capable of determining the analytes of interest precisely over the concentration range encountered. In the analysis of experimental samples, high concentrations of OTC and its epimer were detected in treated bird droppings, which exceeded 2000 μg kg^{-1} on day 14 post the end of treatment. However, concentrations varied between samplings. This fluctuation in the droppings after the end of treatment may be due to the reabsorption or recirculation of OTC in the birds' body to other organs or compartments. Odore et al. [39] detected OTC concentrations in treated broiler chicken bone tissue, this matrix is identified as a target tissue for tetracyclines, were

a more complex link takes place between the tissue, calcium ions, and the rings of the basic tetracycline structure [39]. Moreover, this antibiotic is lipophilic with a large distribution volume, so high concentrations have been detected in different edible tissues, as liver, fat , kidney and muscle [40]. For this reason, and an increase in fat metabolism and recirculation from other tissues, the elimination of OTC could vary over time. Likewise, OTC and 4-epi-OTC quantified in droppings do not correlate with concentrations measured in edible tissues in previous studies, which evaluated the depletion of OTC and 4-epi-OTC residues in muscle, liver, claws and feathers samples from therapeutically treated birds [8,9]. The behavior of CTC and 4-epi-CTC, another tetracycline, has been studied in broiler droppings. CTC + 4-epi-CTC residues of 179.45 μg kg^{-1} 25 days post the end of treatment were measured [41]. The samples showed microbial activity and the presence of resistance genes associated with tetracyclines [42]. Similarly, the concentrations of OTC+4-epi-OTC found in this study are of importance due to the likely selection of resistance in bacteria to these antimicrobials in the gastrointestinal tract of birds [43].

The quantified concentrations of OTC+4-epi-OTC in the litter of treated birds was 10 times greater than that found in droppings day 1 post the end of treatment. Increased concentrations of OTC in litter are likely due to the accumulation of bird droppings throughout the course of the experiment. Sarker et al. [44], analyzed litter samples for the detection of different antimicrobials. They determined that 23% of the samples were OTC positive, with an average concentration of 16.5 mg kg^{-1}. Similar concentrations were observed in this study, whereby at day 7 following the end of treatment with OTC+4-epi-OTC the concentration in litter from the treatment group was 15.6 mg kg^{-1}. We detected a decrease in OTC+4-epi-OTC over time. Then, 14 days post slaughter, the concentrations of these analytes were of 11.4 mg kg^{-1}.

OTC+4epi-OTC persisted in the litter of treated birds post slaughter, at concentrations ranging from 10,360.60 to 15,557.62 μg kg^{-1}. The high concentration of OTC+4-epi-OTC is the result of accumulation of excreted antibiotic during and post the end of treatment. Persistence of OTC+4-epi-OTC in the litter is due to the physical-chemical characteristics of the antibiotic. Kasumba et al. [45] investigated the anaerobic degradation of tetracyclines in the manure of different food-producing animal species and observed a 99-day half-life for OTC in poultry litter. They concluded that the persistence of OTC may be partly explained by the antibiotics ability to form stable complexes with divalent cations, as well as its capacity to adhere to proteins, particles, and organic matter [45].

Our results provide evidence suggesting that droppings may be a route of contamination and dissemination of OTC residues in the environment. Sentinel birds raised adjacent and at a distance of 30 cm from the treated birds, show trace concentrations of OTC in their litter and droppings. However, these concentrations could not be accurately quantified because the results obtained in the samples fluctuated between the LOD and LOQ of method. Detected trace concentrations show a chromatographic response of OTC greater than three times the signal noise of the instrumental baseline (Supplementary Figure S1). At 21 days post the end of treatment there was one positive litter sample in group C with

a concentration of 9489.42 μg kg^{-1} of OTC+4-epi-OTC. It is possible that there was unintentional contamination to the area of the pen where the sample was taken during handling and/or feeding of the birds. In the same way, it could be attributed to the accumulation of OTC in the sampled area.

Movement of the birds and the resulting dust in the air can result in the dissemination of OTC from treated to untreated birds [46]. The long half-life of OTC and the use of broiler droppings to fertilize soil also presents a risk for the transfer and persistence of OTC [47]. Oxytetracycline is known to bind to soil organic matter, clay minerals, and metal oxides [48]. OTC has been detected in soils in concentrations ranging from 0.3 to 300 mg kg^{-1} of soil [49].

Our results show that OTC is excreted from treated birds and can persists in litter, even up to 14 days post slaughter. In this study, a low probability of transfer was observed. This may be mainly due to the administration of the antimicrobial, since it was controlled through the nasogastric tube. Therefore, the factor of loss and dissemination of the antimicrobial through the drinking water or feed was not determined in this study. The carry-over and persistence of this antibiotic may be a risk for the development of antibiotic-resistant pathogens, due to selection pressure [50,51]. For this reason, it is necessary to monitor OTC residue transfer from animals and to investigate potential routes of contamination to help reduce the risk of antibiotic-resistance for both public and animal health.

While the obtained results are interesting and raise some concerns that can be ad-dressed in further studies, some limitations should be recognized. The administration of the drug did not recreate industry conditions since the objective was to ensure the administration of the exact dose. Also, the number of replicates used was the minimum re-quired, in order to analyze more sampling points. Likewise, only one type on litter was study. Therefore, it would be interesting to take these aspects into account in future re-search in order to compare the results obtained and thus determine the factors that may play a role in the persistence and transfer of OTC residues into broiler litter.

5. Conclusion

OTC and 4-epi-OTC in droppings and litter from therapeutically treated broiler chickens were detected following the end of treatment. High concentrations of OTC were excreted from treated birds, ranging from 347.63 μg kg^{-1} to 2244.66 μg kg^{-1} in their droppings. Concentrations of OTC+4-epi-OTC from the litter of treated birds ranged from 10,360.60 to 22,741.68 μg kg^{-1}, and persisted 14 days post slaughter (11,429.14 μg kg^{-1}). However, only trace concentrations of OTC were detected in droppings and litter from sentinel birds. These findings establish the first evidence that there is a low likelihood of the transfer of OTC residues from treated birds to the environment and to untreated birds in adjacent or separate pens, which needs to be further studied.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1. Weights of the experimental birds from group A, which were recorded during the treatment pe-riod, for calculation of the individual dose of oxytetracycline, Table S2. Individual dose of oxytetracycline

administered to birds of group A birds for 10 consecutive days, Table S3. Substance specific mass spectrometric conditions, Table S4. Validation parameters and acceptance criteria following guidelines 2002/657/EC and VICH GL49 for validation of analytical methodology, Table S5. Validation of analytical methodology: Precision and recovery for droppings and litter, Figure S1: Representative OTC chromatograms of the first sampling (1-day post the end of treatment) from samples of (a) droppings from group B; (b) droppings from group C; (c) litter from group B; (d) litter from group C. The chromatographic signal of OTC reaches at least 3 times the signal noise of the baseline.

Author Contributions: Conceptualization, E.P. and J.C.; methodology and validation, E.P., K.Y., L.T., and G.T.; formal analysis and data curation, E.P.; writing—original draft preparation, E.P.; writing—review and editing, J.C., A.M., and N.G.; supervision, J.C., L.L., and B.S.M.; project administration, J.C.; funding acquisition, E.P. and J.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to Law No. 20.380 "On the Protection of Animals", Directive 2010/63/EU on the protection of animals used for scientific purposes and Regulation (EC) No 1099/2009 on the protection of animals at the time of killing and approved by the Institutional Committee for the Care and Use of Animals (CICUA) of the University of Chile (certificate N°: 18187-VET-UCH-E1, approved on 05 July 2019).

Informed Consent Statement: Not applicable

Data Availability Statement: The data presented in this study are available in article.

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CAPÍTULO 2

Evaluación de la resistencia fenotípica y genotípica de cepas de *Escherichia coli* aisladas desde deyecciones y cama de pollos broiler posterior al tratamiento con oxitetraciclina.

1. INTRODUCCIÓN

Los pollos broiler son la principal fuente de carne a nivel mundial, por lo que la industria avícola es una de las industrias de mayor producción (OECD-FAO, 2017). Su nivel de intensificación conlleva a un aumento en el riesgo de contagio de enfermedades infecciosas, por este motivo, la utilización de antimicrobianos ha sido una de las principales herramientas para el tratamiento de las patologías aviares. De esta forma, el control de la presencia de residuos de estos medicamentos veterinarios en los productos y subproductos de la industria avícola es de gran importancia, debido a los efectos adversos que se pueden producir sobre los animales y los consumidores.

Uno de los efectos más importantes, que genera la utilización de antimicrobianos en animales productivos, es la selección de bacterias resistentes. Es en este sentido que se han realizado importantes esfuerzos para controlar el uso indebido o excesivo de estos fármacos, destacándose así, el plan de acción global relativo a la resistencia a los antimicrobianos adoptado en la Asamblea Mundial de la Salud de mayo del año 2015, en donde se establecieron diferentes objetivos relacionados a este tema (OMS, 2016). No obstante, de acuerdo con el informe anual de la Organización Mundial de Sanidad Animal (OIE), publicado en el año 2021, la estimación mundial para el año 2017, sobre el uso de antimicrobianos en función de la biomasa animal aun es importante, registrándose una cantidad de 107,68 mg/kg de peso vivo (OIE, 2021).

A nivel nacional, los antimicrobianos más utilizados para el tratamiento de aves de engorde, y con mayores presentaciones farmacéuticas autorizadas por el Servicio Agrícola y Ganadero (SAG), son las sulfonamidas, macrólidos, lincosamidas y las tetraciclinas. Estas últimas, se encuentran representadas por la oxitetraciclina y la clortetraciclina, y son ampliamente utilizadas en la industria avícola, debido a su buena distribución por el organismo animal y su espectro de acción, el cual abarca una amplia gama de bacterias, que incluyen Gram positivas y Gram negativas (Bryskier, 2005; Grossman, 2016), teniendo un efecto sobre diferentes patologías que afectan a las aves, como el cólera aviar (por

Pasteurella multocida), enfermedad crónica respiratoria (por *Mycoplasma gallisepticum* y *E. coli*), coriza infecciosa (por *Avibacterium paragallinarum*), salmonelosis aviar (*Salmonella* spp.), afecciones digestivas (por enterobacterias), sinovitis infecciosa (por *Mycoplasma synoviae*) y coliseptisemia y onfalitis (por *E. coli*) (SAG, 2021). En Chile existe una formulación de clortetraciclina y cinco de oxitetraciclina de administración oral a través del alimento, autorizadas para su uso en aves de engorde (SAG, 2021).

Los niveles de estos antimicrobianos en los productos comestibles son monitorizados, con la finalidad de asegurar que los residuos no sobrepasen los límites máximos residuales (LMR) establecidos por los diferentes organismos gubernamentales. Estos límites han sido establecidos con la finalidad de que no se produzcan efectos adversos en los consumidores. Sin embargo, la cama de broiler, subproducto de la industria avícola, al no ser destinada para consumo humano directo no presenta LMR establecidos, por lo tanto, su monitorización no es posible (*Codex Alimentarius*, 2017; CE 2010). No obstante, los residuos de antimicrobianos son excretados por las aves y persisten en estos subproductos (Li *et al*., 2013; Hou *et al.,* 2015; Berendsen *et al.,* 2018), por lo que pueden ser un riesgo indirecto para la salud animal y pública. Principalmente porque la cama de broiler, es utilizada como fertilizante agrícola y como materia prima para la elaboración de dietas para otras especies productivas (Divakala *et al.,* 2009).

El uso de antimicrobianos, puede ser un riesgo para la generación y selección de bacterias resistentes, ya que en aves se ha descrito que el uso de estos medicamentos puede generar cambios a nivel de la microbiota intestinal. Xiong *et al.,* (2018) aisló desde aves tratadas bacterias resistentes que presentaron genes de resistencia a múltiples fármacos. Concretamente, las tetraciclinas han dado lugar a la aparición de variantes bacterianas resistentes, en particular las que contienen los genes *tet*, que generalmente están asociados a elementos genéticos móviles o transposones conjugativos (Chopra y Roberts 2001; Roberts 2005; Thaker *et al.,* 2010), los cuales codifican para diferentes mecanismos de resistencia contra estos antimicrobianos, como las bombas de eflujo, protección ribosomal, inactivación enzimática y diferentes mutaciones, como la descrita en la subunidad ribosomal 30S (Bryskier, 2005). Actualmente se argumenta que los genes son contaminantes emergentes, debido a que han sido detectados en diferentes compartimentos ambientales, incluyendo suelos, sedimentos de ríos, causes de agua y aguas residuales (Pruden, 2006; Zalewska, 2021). Su presencia en los diferentes ambientes es un riesgo debido a que

pueden ser adquiridos por otras bacterias patógenas que afecten a humanos y animales (Zhao *et al.,* 2017; Xiong *et al.,* 2018).

Escherichia coli es un bacilo Gram negativo que forma parte de la microbiota intestinal normal de animales y humanos. Sin embargo, existen algunas cepas patogénicas que pueden causar enfermedades mortales en humanos, mamíferos y aves (Etcheverría *et al.,* 2016). Estas bacterias son utilizadas como bacterias indicadoras de niveles de resistencia a los antimicrobianos en diferentes especies productivas, ya que se ha descrito que son un reservorio de genes de resistencia, los cuales podrían ser transmitidos a bacterias patógenas y zoonóticas (Moreno *et al.,* 2000; Phuc Nguyen *et al.*, 2009; Echeverría *et al.,* 2016; Agyare *et al*., 2018). Se ha observado que la administración de antimicrobianos aumenta el riesgo de resistencia a estos fármacos en *E. coli* en cerdos. No obstante, existe una carencia en el estudio de las dosis o concentraciones, y los efectos a lo largo del tiempo (Burow *et al.,* 2014).

En cuanto a la relación entre la presencia de residuos de antibióticos y prevalencia de resistencia en medicina veterinaria, Chantziaras *et al.* (2014) evaluaron la correlación entre el uso de antibióticos y la prevalencia de cepas de *E. coli* aisladas a partir de cerdos, aves de corral y bovinos. Sus resultados indicaron que existe una correlación entre el uso de antimicrobianos específicos y el nivel de resistencia de estos microorganismos. Sin embargo, existen muchas restricciones de datos en este estudio, por lo que ellos concluyen que es necesario un mayor detalle en la recopilación y armonización de estos.

Actualmente no se tiene conocimiento de estudios controlados que determinen el efecto de la administración de OTC, a dosis terapéuticas, en pollos de engorde, sobre la selección de cepas resistentes post-tratamiento. Por este motivo, el objetivo del presente estudio fue determinar la presencia de aislados de *E. coli* no susceptibles a este antibiótico en deyecciones y cama de broiler de aves tratadas y no tratadas, para así definir si existe una relación entre el tratamiento y la selección de bacterias resistentes en estas matrices de estudio.

2. MATERIAL Y MÉTODOS

2.1. Diseño experimental del estudio in vivo

2.1.1. Animales experimentales

Para el estudio *in vivo* se trabajó con pollos broiler machos de la línea genética Ross 308 (Ross®, Aviagen Inc., Huntsville, AL, EE.UU). Las aves se criaron desde el primer día de vida en una unidad experimental especialmente diseñada para llevar a cabo este estudio. En este experimento se acondicionaron corrales de 1,5 m^2 con viruta limpia y analizada previamente (análisis químico y microbiológico), ya que esta viruta posteriormente formó parte de la cama de las aves. Se controlaron las condiciones ambientales, como la temperatura, de acuerdo con los requerimientos de la edad, la humedad (50-60%) y la ventilación.

Durante el experimento, las aves tuvieron acceso *ad libitum* al agua y al alimento no medicado. Las dietas se formularon de acuerdo con los requerimientos nutricionales de la raza, tal y como se recomienda en el manual de Aviagen™ (Aviagen Ross, 2019).

El protocolo de manejo y seguimiento de las aves de experimentación se basó en la Ley 20.380 "Sobre la protección de los animales" y en la Directiva 2010/63/UE sobre la protección de los animales utilizados con fines científicos (UE, 2010). Se respetó el Reglamento (CE) nº 1099/2009 sobre la protección de los animales en el momento del sacrificio (Ministerio de Salud, 2009). El experimento fue aprobado por el Comité Institucional de Cuidado y Uso de Animales (CICUA) de la Universidad de Chile, certificado N°: 18187-VET-UCH-E1.

2.1.2. Grupos experimentales y tratamiento de aves

Desde el primer día de vida las aves fueron aleatoriamente agrupadas en tres grupos experimentales. El grupo A incluía seis aves tratadas con una formulación farmacéutica que contenía OTC a 100 mg mL^{-1} (10%), cuyo uso está autorizado en aves de engorde. Todas las aves del grupo A fueron tratadas por vía oral mediante una sonda orogástrica (Levin nº6) y una jeringa estéril, con una dosis terapéutica de 80 mg kg^{-1} durante 10 días consecutivos, de acuerdo con las indicaciones de la etiqueta. El fármaco se administró directamente en el buche, según el peso individual asegurando la entrega de las dosis calculadas.

El segundo y tercer grupo (Grupos B y C) también conformados por seis aves cada uno, no fueron tratados, el grupo B se mantuvo en un corral adyacente al grupo A, mientras que el grupo C se mantuvo a 30 cm del grupo A. En este último se determinó una separación efectiva de 30 cm para evitar el contacto directo total entre las aves. Todos los corrales

tenían una superficie de 1,5 m^2, dividida por una pared sólida de 1,5 m de altura. Se tomaron todas las medidas para evitar la contaminación por manipulación, como el uso de cubre calzado y guantes para la manipulación de cada grupo experimental.

2.1.3. Toma de muestras fecales y de cama de broiler

Se tomaron muestras de deyecciones de cada ave mediante torulado cloacal, las cuales fueron dispuestas en tubos de vidrio estériles. Las muestras de cama de broiler fueron obtenidas a partir de cada corral y almacenadas en bolsas plásticas estériles. Se obtuvieron seis muestras de 10 g de cada corral de acuerdo con el protocolo de muestreo de suelos del Servicio Agrícola y Ganadero (SAG), el cual establece los requisitos de homogeneidad según el artículo 28 del DS: nº 4/09 (SAG, 2019). De acuerdo con esto, las muestras se recolectaron de manera equidistante dentro de cada corral, cubriendo toda el área, utilizando un método de muestreo asistemático, tal como lo sugiere el protocolo previamente mencionado.

Para le aislamiento de *E. coli,* las muestras fueron procesadas inmediatamente y los puntos de muestreo correspondieron a los días 1, 7, 14 y 21 después de finalizado el tratamiento. También se analizaron muestras de cama, una y dos semanas después del sacrifico de las aves.

2.2. Aislamiento y procedimiento de identificación de E. coli desde muestras fecales y cama de broiler

2.2.1. Medios de cultivo y reactivos

Para el aislamiento e identificación de *E. coli* a partir de las muestras experimentales se utilizaron los siguientes medios de cultivo y reactivos: agar MacConkey, agua de peptona tamponada (APT), agar movilidad-indol-ornitina (MIO), preparado con extracto de Levadura (3 g), peptona (10 g), triptona (10 g), L-ornitina (5 g), dextrosa (1 g), purpura de bromocresol (0,02 g), agar (2 g) en 1 L de agua destilada. Medio Voges-Proskauer (preparado con peptona (7 g), glucosa (5 g), K_2HPO_4 (5 g), en 1 L de agua destilada), citrato de Simmons, Rojo de metilo, etanol al 95% grado analítico, hidróxido de potasio y α-naftol.

2.2.2. Aislamiento de *E. coli* desde muestras fecales y cama de broiler

Todas las muestras se analizaron inmediatamente después del muestreo. Se pesó un 1 g de muestra de cama y se adicionó 9 mL de APT. En el caso de los torulados cloacales, a estos se les adicionó 4,5 mL de APT. Posteriormente las muestras fueron homogenizadas y sembradas por agotamiento de estrías en agar MacConkey. Las colonias rosas, redondas y de tamaño medio se seleccionaron como colonias sospechosas de *E. coli* y fueron sometidas a pruebas IMVic.

2.2.3. Identificación de *E. coli* mediante prueba IMViC

La identificación de *E. coli* previamente aisladas fue mediante la prueba IMViC, que consiste en cuatro pruebas: prueba de producción indol, prueba de Voges-Proskauer, rojo de metilo y prueba de agar citrato de Simmons (Lupindu, 2017; Nkogwe, *et al*, 2011).

Para la prueba de producción de indol, se inoculó con asa de punta de manera vertical en agar MIO y se incubó a 35°C ± 1 por 24 ± 2 horas. Una vez finalizada la incubación se añadió 0,2 a 0,3 mL de reactivo de Kovacs. Para confirmación de *E. coli* se espera una reacción positiva para el biotipo I, que consiste en la formación de un anillo rojo intenso en la superficie del medio. Por su parte, la reacción negativa se da en cepas de *E. coli* del biotipo II, la cual es la presencia de un anillo de color amarillo.

En este mismo medio se evaluó la motilidad, y para cepas de *E. coli* se consideró una reacción positiva cuando se observó un crecimiento más allá de la línea de inoculación y turbidez en el medio. Asimismo, se evaluó la descarboxilación de la ornitina, en donde el color morado marco la reacción positiva para *E. coli.*

La prueba de Voges-Proskauer, se realizó mediante la inoculación del medio del mismo nombre y se incubó a 35°C ± 1 por 24 ± 2 horas. Una vez finalizada la incubación, 1 mL del cultivo se trasladó a otro tubo y se adicionaron 0,6 mL de α-Naftol y 0,2 mL de KOH al 40%, se agitó y se incubó por 2 horas a 35°C ± 1. *E. coli* presenta una reacción negativa para esta prueba, la que consta de una coloración parda.

La prueba Rojo de Metilo (RM), se realizó utilizando el cultivo restante de Voges-Proskauer al cual se le agregaron 5 gotas de indicador de rojo de metilo al 0,02%. La coloración roja corresponde a una reacción positiva en esta prueba y la coloración amarilla a una reacción negativa. En esta prueba *E. coli* da positiva.

La prueba de Agar citrato de Simmons, se realizó inoculando e incubando en este medio los aislados a 35°C ± 1 por 24 ± 2 horas. La prueba de utilización de citrato detecta la capacidad de las bacterias para usar el citrato como su única fuente de carbono y energía, el citrato en los medios se descompone en oxalacetato y acetato debido a la acción de una enzima citritasa. *E. coli* no utiliza el citrato como fuente de energía, por lo que la reacción negativa es confirmatoria de *E. coli*, correspondiendo a una coloración verde sin desarrollo bacteriano. Por su parte, las muestras con desarrollo bacteriano y coloración azul no fueron confirmadas como *E. coli.*

2.3. Análisis de susceptibilidad de las cepas y su relación con los grupos experimentales.

2.3.1. Medios de cultivo y disco antimicrobiano

El caldo soya tripticasa (TSB), el cloruro de sodio (NaCl), agar Mueller Hinton (M-H), agar nutritivo y disco antimicrobiano con tetraciclina 30 µg (OXOID®), fueron requeridos para las pruebas de sensibilidad.

2.3.2. Método de difusión en disco Kirby-Bauer

Con un asa circular se tomaron de 4 a 5 colonias de cultivos puros y se incubaron a 35°C por 18 horas en TSB (Hudzicki, 2009). Posterior a esto se comprobó la turbidez de la suspensión bacteriana en un espectrofotómetro (Halo RB-10, Dynamica Ltd., Kuala Lumpur, Malasia) y se ajustó la concentración de la suspensión mediante diluciones en solución salina a 0,85%, hasta alcanzar un DO entre 0,08 y 0,1 a 600 nm, lo que equivale a 0,5 McFarland.

Luego de ajustada la turbidez, se inoculó el agar utilizando un hisopo estéril. Se giró la placa en 60° y se repitió el paso dos veces, para obtener una distribución uniforme del inóculo. Se dispuso un disco antimicrobiano en la placa posterior a 15 minutos de inoculada la placa y se presionó ligeramente el disco hacia abajo para asegurar el contacto con la superficie del agar. Las placas se incubaron invertidas a 35°C durante 16-18 horas. La zona de inhibición se observó después de la incubación y se midió el diámetro de los halos de inhibición con un pie de metro. Se comparó el diámetro de la zona de inhibición de los aislamientos de prueba con los criterios de interpretación del documento del *Clinical and Laboratory Standards Institute* (CLSI, 2015).

2.3.3. Análisis estadístico

Se realizó el análisis estadístico descriptivo para las variables cualitativas mediante el cálculo de frecuencias absolutas y relativas. En el caso de las variables cuantitativas (concentración en µg kg^{-1} de OTC + 4-epi-OTC), se realizó análisis de los parámetros de posición y dispersión mediante la media de los datos y las desviaciones estándares obtenidas. Estos datos fueron publicados previamente (Pokrant *et al.,* 2021). Para determinar la independencia entre los grupos, se realizó la prueba no paramétrica de independencia de Chi-cuadrado, donde los criterios de clasificación fue la susceptibilidad y la condición (tratado/no tratado), y las frecuencias correspondieron a los porcentajes de aislados no susceptibles y susceptibles. El análisis se llevó a cabo mediante el software InfoStat versión 2020I. Se consideró diferencia estadísticamente significativa cuando el *p* valor < 0,05, rechazando la hipótesis nula de independencia.

2.4. Análisis molecular de cepas no susceptibles

2.4.1. Reactivos y partidores

Agua ultrapura, tampón TAE 1x, agarosa, agua libre de nucleasas, master mix (que contiene: ADN Polimerasa, dNTPs, $MgCl_2$ y tampones de reacción) (GoTaq® G2 grenn master mix,

Promega, WI, USA), partidores de genes *tet*, los cuales se describen en la tabla 1. Las soluciones stock de los partidores se prepararon a una concentración de 100 μM y la solución de trabajo a una concentración de 10 μM.

Tabla 1. Secuencia de los partidores, temperatura de fusión y tamaño del amplicón para cada gen de resistencia a tetraciclinas (genes *tet*) en estudio.

Gen	Secuencia (5'>3')	Tm* (50 mM NaCl) (℃)	Tamaño (pb**)	Referencia
***tet*(A)**	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	56.2 55,1	210	Ng *et. al.,* 2001
***tet*(B)**	F: ACGGARAGTTTATTGTATACC R: TGGCGTATCTATAATGTTGAC	53,5 53,9	659	Ng *et. al.,* 2001
***tet*(C)**	F: TTGGTTAGGGGCAAGTTTTG R: GTAATGGGCCAATAACACCG	55,6 56,6	418	Ng *et. al.,* 2001
***tet*(D)**	F: GGAATATCTCCCGGAAGCGG R: CACATTGGACAGTGCCAGCAG	53,8 54,9	787	Ng *et. al.,* 2001
***tet*(E)**	F: CTTGAGAGCCTTCAACCCAG R: ATGGTCGTCATCTACCTGCC	55,1 55,3	278	Ng *et. al.,* 2001
***tet*(M)**	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	53,3 53,8	406	Ng *et. al.,* 2001
***16s*RNA**	F: GACCTCGGTTTAGTTCACAGA R: CACACGCTGACGCTGACCA	54,1 60,6	585	Mamun *et al*., 2016
E. coli uspA	F: CCGATACGCTGCCAATCAGT R: ACGCAGACCGTAGGCCAGAT	57,6 61,0	884	Toro *et al.,* 2018

*Tm: Temperatura de fusión del partidor; **pb: pares de bases

2.4.2. Preparación de ADN bacteriano

Se tomó una asada, a partir de las cepas previamente sembradas e incubadas en agar MacConkey a 37°C durante 18-24 horas, y se inoculó en 500 µL de agua estéril libre de nucleasas. Luego se llevó a ebullición durante 15 min a 100°C, se centrifugó a 26.480 g durante 5 min a temperatura ambiente. El ADN extraído se cuantificó con un espectrofotómetro (microespectrofotómetro NANO-400, Hangzhou Allsheng instruments Co.). Las muestras que presentaron una relación de absorbancia de 260/280 nm cercana al rango óptimo (1,8-2,0) se analizaron mediante PCR.

2.4.3. Determinación de genes *tet* mediante PCR convencional

Para determinar los genes de resistencia presentes en los aislados, se realizó un PCR convencional utilizando el ADN extraído como templado. Los genes a analizados correspondieron a genes *tet*, específicamente *tet*(A), *tet*(B), *tet*(C), *tet*(D), tet(E) y tet(M); el gen 16S rRNA fue incluido para la confirmación de presencia de ADN y el gen *uspA* para confirmación de la identidad de *E. coli* (Toro *et al.,* 2018). Las condiciones y partidores se describen en la tabla 1. Como control negativo se utilizó la cepa *E. coli* ATCC® 25922, y

como control positivo se utilizó el ADN de cepas que fueron previamente secuenciadas y que presentan los genes objetivo.

La amplificación del ADN se llevó a cabo en un termociclador LifeECO (Hangzhou Bioer Technology Co. Ltd. Zhejiang, China) utilizando las siguientes condiciones: una desnaturalización inicial de 5 minutos a 94°C, seguida de 35 ciclos de 94°C durante 1 minuto, 54°C durante 1 minuto y 72°C durante 1-5 minutos. Finalmente, 5 μL del producto de PCR se visualizó en gel de electroforesis (agarosa al 2% (p/v) en tampón 1×TAE), previamente teñido con Safeview®. Las bandas se visualizaron mediante transiluminación ultravioleta y los tamaños de los productos de la PCR se determinaron comparándolos con la escala de un tamaño de 100 pares de bases (pb) (Maestrogen Hsinchu, Taiwán).

2.5. Tipificación en cepas no susceptibles

2.5.1. Medios y Reactivos

SeaKem Gold" al 1% (Lonza, Rockland, ME USA) y SDS al 1%, proteinasa K (Invitrogen, Waltham, MA USA), enzima de restricción XbaI (Thermo-Fisher Scientific, Waltham, MA USA), 45 mM de Tris-HCl, 45 mM de ácido bórico, 1 mM de ácido etilendiaminotetraacético (EDTA).

2.5.2. PFGE de cepas de *E. coli* resistentes a tetraciclina

Para determinar la diversidad genética de las cepas de *E. coli* aisladas desde las muestras de deyecciones y cama de broiler, se realizó una tipificación mediante electroforesis en gel en campo pulsado (PFGE). Se procesaron 60 cepas de *E. coli* resistentes, correspondientes al muestreo previo al tratamiento y al primer punto de muestreo una vez finalizado el tratamiento, debido a que es este último es el muestreo más representativo del efecto del tratamiento. El análisis se realizó de acuerdo con lo establecido en *"Standard Operating Procedure for PulseNet PFGE of Escherichia coli O157:H7, Escherichia coli non- O157 (STEC), Salmonella serotypes, Shigella sonnei and Shigella flexneri"* (CDC, 2021) con algunas modificaciones. Se generó una suspensión bacteriana de 0,4 de DO a 420 nm. Se prepararon bloques con agarosa "SeaKem Gold" al 1% (Lonza, Rockland, ME USA) y SDS al 1%. Las células incluidas en el bloque fueron lisadas con 1mg/mL de proteinasa K (Invitrogen, Waltham, MA USA) por 3 horas a 55ºC. Posteriormente el genoma fue digerido con 50 U de la enzima de restricción XbaI (Thermo-Fisher Scientific, Waltham, MA USA) a 37ºC por 3 horas. Los bloques de agarosa se cargaron en un gel de agarosa "Pulse Field Certified" al 1% (Bio-Rad, Hercules, CA USA) con tampón TBE al 0,5% (45 mM Tris-HCl, 45 mM ácido bórico, 1 mM EDTA). La electroforesis se realizó en un equipo CHEF-DR III system (Bio-Rad) a 14°C por 20 horas, 6 V/cm con un pulso inicial de 2,16 s y un pulso final

de 54,17 s. En cada gel se incluyeron bloques de la cepa de referencia *Salmonella braenderup* H9812, los cuales fueron tratados de la misma forma anteriormente descrita, con el fin de permitir la normalización de cada gel. Finalmente, los perfiles de las 60 cepas y de la cepa de *Salmonella braenderup*, fueron analizadas con el programa “Gel Compar II” versión 5.10 (Applied Maths, Sint-Martens-Latem, Belgica). Los dendrogramas se generaron utilizando el coeficiente de similitud de Dice basado en las bandas y el método UPGMA, con un 1% de tolerancia en la posición de las mismas (Heng *et al.,* 2009; Matsumoto *et al.,* 2011; Bakuła *et al.,* 2018). Los patrones moleculares obtenidos por XbaI-PFGE fueron agrupados en conjuntos clonales cuando presentaron una similitud del 100%. Se fijó un valor de corte del 80% para determinar la similitud de los clusters (Luizaga de Monteiro *et al.,* 2013, Rivoal *et al.,* 2013).

2. RESULTADOS

3.1. Muestras de E. coli aisladas desde deyecciones y cama de broiler.

Las muestras de deyecciones y cama fueron analizadas previo al experimento y se aisló *E. coli* para determinar el perfil de resistencia antes del tratamiento. Solo a partir de las muestras de deyecciones se asiló *E. coli,* y de las 90 colonias aisladas 80 se confirmaron mediante las pruebas bioquímicas. Por su parte, en las muestras de viruta, que correspondieron al sustrato de las camas de las aves, no se observó crecimiento de microorganismo después de su siembra en los agares selectivos para enterobacterias.
En cambio, desde las diferentes muestras analizadas a partir de los muestreos post-tratamiento, se aislaron colonias de *E. coli*, las cuales también fueron confirmadas mediante pruebas bioquímicas. Se analizaron seis muestras de deyecciones y seis muestras de cama por cada grupo experimental a los días 1, 7, 14 y 21 post-tratamiento. Adicionalmente, se muestrearon muestras de cama post-faena a los 7 y 14 días después de que los corrales permanecieron desocupados (equivalente a los días 29 y 36 post-tratamiento). De las colonias típicas en agar MacConkey se obtuvieron cinco colonias de cada muestra y se confirmaron mediante la prueba IMViC (Figura suplementaria 1). Se confirmaron las cepas que presentaron resultados positivos a la prueba Ornitina, motilidad y rojo de metilo, y resultados negativos a la prueba Voges-Proskauer y citrato. Por su parte, los resultados positivos y negativos fueron considerados para la prueba de Indole (con reactivo de Kovacs), ya que el biotipo I presenta una reacción positiva y el biotipo II una reacción negativa a esta prueba. En la tabla 2 se registra el número de aislados confirmados según muestreo, matriz y grupo experimental.

Tabla 2. Colonias de *E coli* confirmadas mediante prueba IMViC desde muestras de deyecciones y camas de broiler, obtenidas en los distintos puntos de muestreo (días postratamiento).

Número de aislados de *E. coli* confirmados por prueba IMViC						
Días*	**Grupo tratamiento**		**Grupo Centinela Adyacente al tratamiento**		**Grupo Centinela a 30 cm del tratamiento**	
	Deyecciones	**Cama**	**Deyecciones**	**Cama**	**Deyecciones**	**Cama**
1	20	29	22	30	13	30
7	19	12	9	19	17	15
14	22	18	21	20	12	15
21	23	17	9	11	14	10
29	-	22	-	20	-	10
36	-	25	-	16	-	25

Grupo Tratamiento: animales tratados con una formulación de OTC 10%; Grupo centinela adyacente y a 30 cm del tratado, mantenido bajo las mismas condiciones experimentales, pero sin tratamiento.
* Días Post-tratamiento.

3.2. Perfiles de resistencia de cepas de E. coli aisladas a partir de las muestras experimentales y su relación con los grupos experimentales.

La totalidad de los aislados que fueron confirmados como *E. coli*, se analizaron mediante la prueba Kirby-Bauer. En la figura 1 se muestran imágenes representativas de cepas sensibles y resistentes a discos de tetraciclina de 30 μg. Este disco fue utilizado de acuerdo con las recomendaciones del CLSI 3^{era} edición para el análisis de sensibilidad de las cepas (CLSI, 2015). En este documento se indica que, para Enterobacterias, el antimicrobiano tetraciclina es testeado como el representante de la clase de susceptibilidad a clortetraciclina, doxiciclina, minociclina y oxitetraciclina. Los organismos que son susceptibles a tetraciclina también se consideran susceptibles a doxiciclina y minociclina. Sin embargo, algunos organismos que son intermedios o resistentes a la tetraciclina pueden ser susceptibles a la doxiciclina, a la minociclina o a ambas (CLSI, 2015).

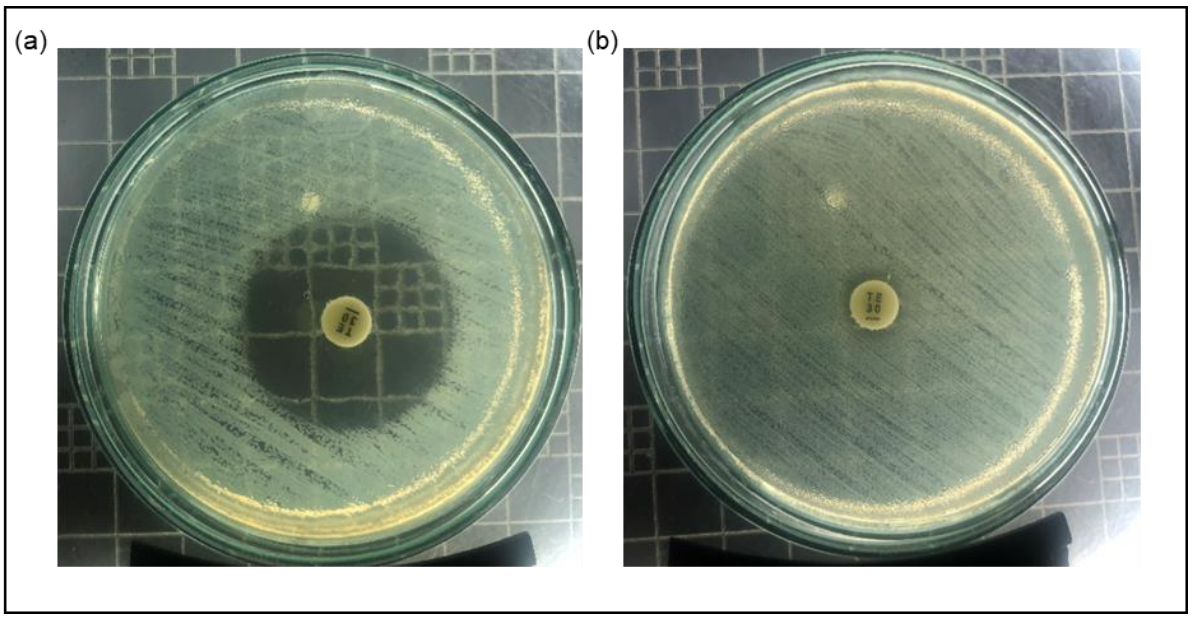


Figura 1. Imagen representativa del análisis mediante el método de difusión en disco (Kirby- Bauer) de cepas de *E. coli*, aisladas desde muestras de deyecciones y cama de pollos broiler. La sensibilidad fue determinada de acuerdo con el diámetro de los halos de inhibición bacteriana, según criterios de CLSI, 2019 para enterobacterias y discos de tetraciclina a 30 µg. (Resistente: ≤11 mm; Intermedia: 12-14 mm; Sensible: ≥ 15 mm). Imagen izquierda: cepa sensible, con un diámetro de 30 mm; Imagen derecha: cepa resistente sin halo de inhibición.

A partir de los análisis, se detectaron cepas sensibles y resistentes en todos los puntos de muestreo para las dos matrices de estudio. También, se detectaron cepas con sensibilidad intermedia, principalmente en los muestreos de cama post-faena, donde se determinó una mayor proporción de estos aislados (Tabla 3 y 4). Los muestreos post-faena correspondieron a los días 7 y 14 posterior al sacrificio de las aves, es decir los corrales fueron muestreados cuando se encontraban deshabitados por un periodo de 1 y 2 semanas, por lo que no existió un aporte de material fecal y las condiciones de temperatura y humedad fueron las ambientales. Los residuos de OTC y su epímero en cada de una de las muestras fueron cuantificadas previamente (Pokrant *et al*., 2021) y se compararon con el porcentaje de cepas no susceptibles aisladas a partir de las mismas muestras y los distintos grupos experimentales. En la tabla 3 y 4, se observa que el mayor porcentaje de cepas no susceptibles se detectaron en las muestras del grupo tratamiento, tanto en deyecciones como cama.

Tabla 3. Porcentaje de aislados resistentes/sensibilidad intermedia (nos susceptibles), y concentraciones de OTC + 4-epi-OTC detectadas en deyecciones de las aves, según grupo experimental a lo largo de los días postratamiento.

Días	Grupo tratamiento		Grupo Centinela Adyacente al tratamiento		Grupo Centinela a 30 cm de tratamiento	
	No susceptibles (%)	Concentración µg Kg^{-1}	No susceptibles (%)	Concentración µg Kg^{-1}	No susceptibles (%)	Concentración µg Kg^{-1}
1	100 (R)	2.087,41	4,6 (R)	<LOD	76,9 (R)	<LOD
7	84,2 (R)	347,63	22,2 (R)	<LOD	41,2 (R)	<LOD
14	54,5 (R) / 9,1 (I)	2.244,66	23,8 (R)	<LOD	16,7 (R)	N/D
21	17,4 (R) / 17,4 (I)	733,00	22,2 (R) / 11,1 (I)	<LOD	28,6 (R)	N/D

<LOD: bajo el límite de detección de la técnica analítica (12,5 µg kg^{-1}). N/D: no detectado; R: Resistentes; I: Sensibilidad intermedia

Tabla 4. Porcentaje de aislados bacterianos resistentes/sensibilidad intermedia (no susceptibles), y concentraciones de OTC + 4-epi-OTC detectadas en cama, según grupo experimental a lo largo de los días postratamiento.

Días	Grupo tratamiento		Grupo Adyacente al tratamiento		Grupo a 30 cm de tratamiento	
	No susceptibles (%)	Concentración µg Kg^{-1}	No susceptibles (%)	Concentración µg Kg^{-1}	No susceptibles (%)	Concentración µg Kg^{-1}
1	82,8 (R)	22.741,68	16,7 (R)	<LOD	6,7 (R)	<LOD
7	66,7 (R) / 8,3 (I)	15.594,05	10,5 (R) / 5,3 (I)	<LOD	S/D	<LOD
14	27,8 (R) / 44,4 (I)	12.236,68	5,0 (R)	<LOD	S/D	<LOD
21	11,8 (R) / 5,9 (I)	12.946,13	9,1 (R)	<LOD	20,0 (R) / 10,0 (I)	<LOD
29*	45,5 (R) / 36,4 (I)	15.557,62	55,0 (R) / 15,0 (I)	<LOD	30,0 (R)	<LOD
36*	65,0 (R) / 28,0 (I)	11.429,14	25,0 (R) / 25,0 (I)	<LOD	8,0 (R)	<LOD

* días post-faena, correspondientes a 7 y 14 días después del sacrificio de los animales experimentales; <LOD: bajo el límite de detección de la técnica analítica (12,5 µg kg^{-1}); R: Resistentes; I: Sensibilidad intermedia; S/D: Sin detección.

Para el análisis estadístico no paramétrico de independencia de Chi-cuadrado, se obtuvieron *p* valores de < 0,0001 para los resultados obtenidos a partir de los análisis por matriz (deyecciones y cama de broiler). Como el valor obtenido fue menor al nivel de significancia de 0,05 seleccionado, se rechazó la hipótesis nula a favor de la dependencia entre susceptibilidad y condición de tratamiento de los grupos estudiados.

3.3. Determinación de genes tet y grupos filogenéticos de cepas de E. coli resistentes.

3.3.1. Análisis de genes de resistencias presentes en muestras de deyecciones y cama.

50 cepas de *E. coli* no susceptibles provenientes de las muestras de deyecciones y cama del primer punto de muestreo (día 1 post-tratamiento) y 10 cepas de *E. coli* del muestreo previo al tratamiento, fueron analizadas mediante PCR convencional. Estos aislados, fueron confirmados previamente como *E. coli* mediante la detección del gen *uspA* (Toro et al., 2018). En la figura 2 se observan los productos de PCR de un tamaño aproximado de 900 pb, lo que corresponde a la amplificación del gen *uspA* de 884pb.

Se determinó que las cepas de *E. coli* resistentes previo al tratamiento solo tenían genes *tet*(A). En cambio, los aislados provenientes del primer día post-tratamiento, tenían genes *tet(*A), *tet(*B), *tet*(C) y *tet*(M). Donde un 80% de los aislados tenia a genes *tet*(A), un 12% genes *tet*(B), un 10% genes *tet*(C) y un 14% genes *tet*(M). En la figura 2 se observan los productos de PCR.

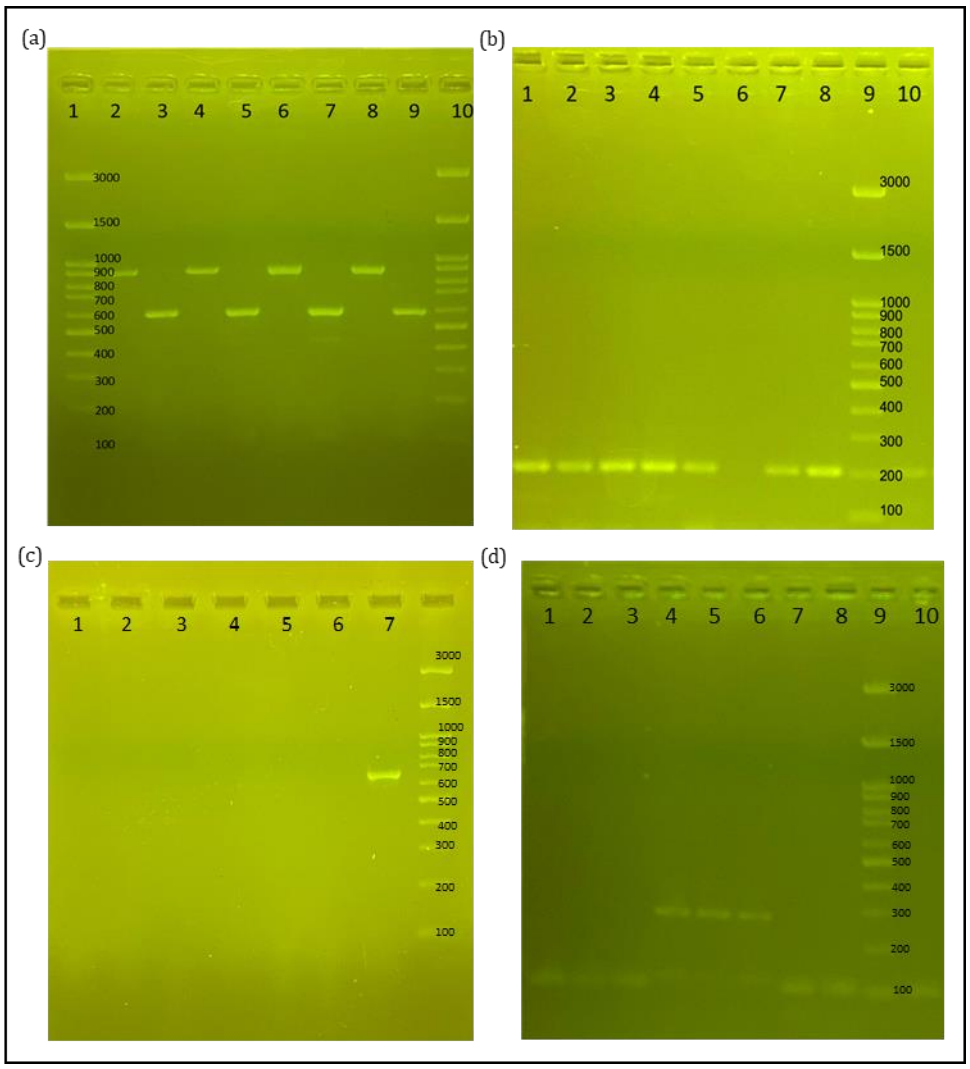
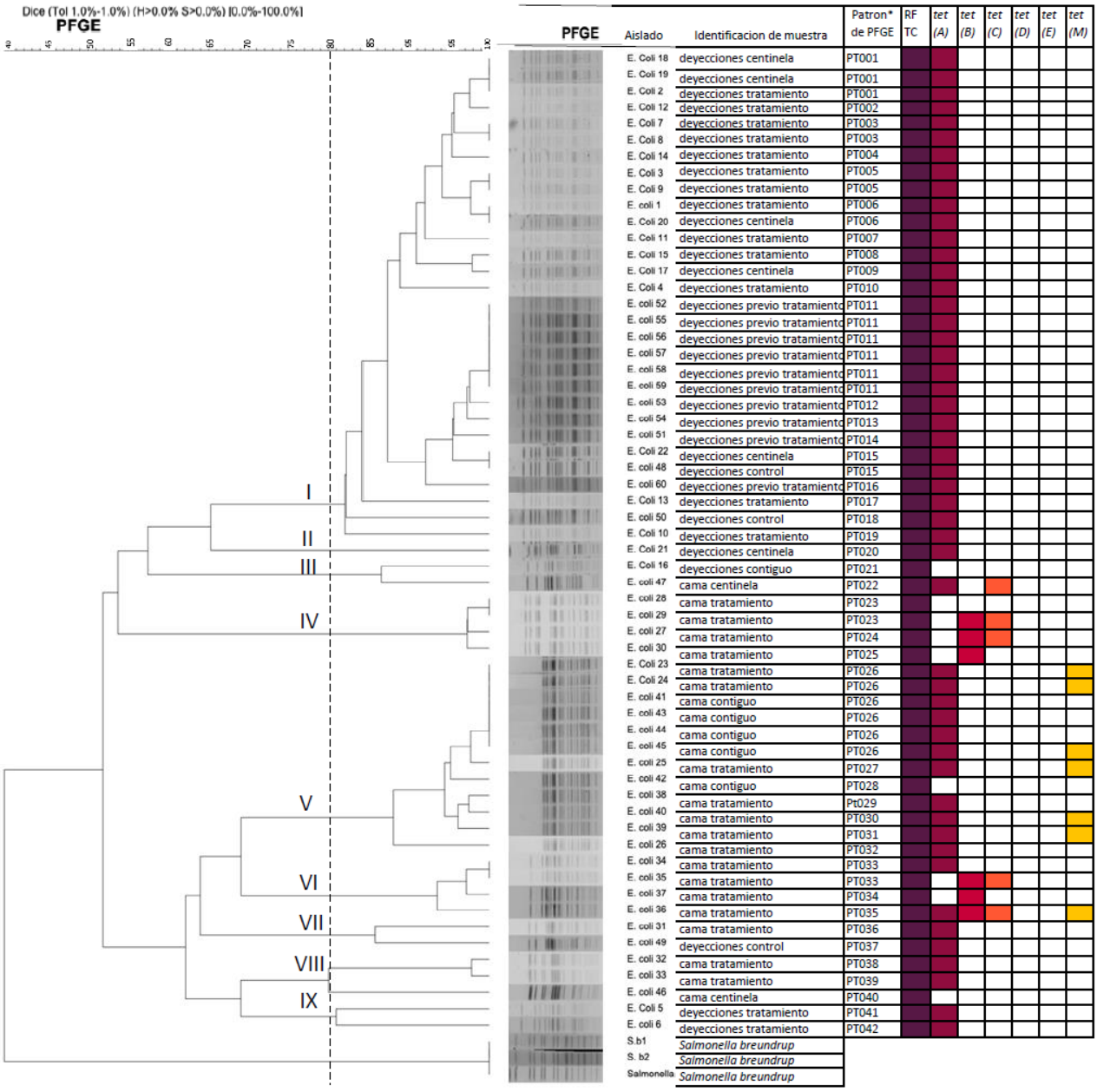


Figura 2. Imagen representativa de aislados de *E. coli*, desde muestras correspondientes al primer punto de muestreo (día 1 postratamiento) confirmados por PCR, mediante la detección del (a) gen *uspA* de un tamaño de 884 pb y el gen 16sRNA con un tamaño de 585 pb, (b) gen *tet*(A) con un tamaño de 210 pb, (c) gen *tet*(B) con un tamaño de 659 pb y (d) gen *tet*(M) con un tamaño de 406 pb.

Posterior al análisis de PFGE y generación del dendrograma, se definieron nueve clusters considerando como punto de corte un 80% de similitud. Los clusters I, II, III y IX, corresponden casi exclusivamente a aislados provenientes desde muestras de deyecciones, a excepción de un aislados de cama que se incluye en el cluster III. Por su parte, todos los otros aislados obtenidos a partir de las muestras de cama se agruparon en los clusters IV, V, VI, VII y VIII (Figura 3).



PFGE	Aislado	Identificacion de muestra	Patron* de PFGE	RF TC	*tet (A)*	*tet (B)*	*tet (C)*	*tet (D)*	*tet (E)*	*tet (M)*
	E. Coli 18	deyecciones centinela	PT001							
	E. Coli 19	deyecciones centinela	PT001							
	E. Coli 2	deyecciones tratamiento	PT001							
	E. Coli 12	deyecciones tratamiento	PT002							
	E. Coli 7	deyecciones tratamiento	PT003							
	E. Coli 8	deyecciones tratamiento	PT003							
	E. Coli 14	deyecciones tratamiento	PT004							
	E. Coli 3	deyecciones tratamiento	PT005							
	E. Coli 9	deyecciones tratamiento	PT005							
	E. coli 1	deyecciones tratamiento	PT006							
	E. Coli 20	deyecciones centinela	PT006							
	E. Coli 11	deyecciones tratamiento	PT007							
	E. Coli 15	deyecciones tratamiento	PT008							
	E. Coli 17	deyecciones centinela	PT009							
	E. Coli 4	deyecciones tratamiento	PT010							
	E. coli 52	deyecciones previo tratamiento	PT011							
	E. coli 55	deyecciones previo tratamiento	PT011							
	E. coli 56	deyecciones previo tratamiento	PT011							
	E. coli 57	deyecciones previo tratamiento	PT011							
	E. coli 58	deyecciones previo tratamiento	PT011							
	E. coli 59	deyecciones previo tratamiento	PT011							
	E. coli 53	deyecciones previo tratamiento	PT012							
	E. coli 54	deyecciones previo tratamiento	PT013							
	E. coli 51	deyecciones previo tratamiento	PT014							
	E. Coli 22	deyecciones centinela	PT015							
	E. coli 48	deyecciones control	PT015							
	E. coli 60	deyecciones previo tratamiento	PT016							
	E. Coli 13	deyecciones tratamiento	PT017							
	E. coli 50	deyecciones control	PT018							
	E. Coli 10	deyecciones tratamiento	PT019							
	E. Coli 21	deyecciones centinela	PT020							
	E. Coli 16	deyecciones contiguo	PT021							
	E. coli 47	cama centinela	PT022							
	E. coli 28	cama tratamiento	PT023							
	E. coli 29	cama tratamiento	PT023							
	E. coli 27	cama tratamiento	PT024							
	E. coli 30	cama tratamiento	PT025							
	E. Coli 23	cama tratamiento	PT026							
	E. Coli 24	cama tratamiento	PT026							
	E. coli 41	cama contiguo	PT026							
	E. coli 43	cama contiguo	PT026							
	E. coli 44	cama contiguo	PT026							
	E. coli 45	cama contiguo	PT026							
	E. coli 25	cama tratamiento	PT027							
	E. coli 42	cama contiguo	PT028							
	E. coli 38	cama tratamiento	Pt029							
	E. coli 40	cama tratamiento	PT030							
	E. coli 39	cama tratamiento	PT031							
	E. coli 26	cama tratamiento	PT032							
	E. coli 34	cama tratamiento	PT033							
	E. coli 35	cama tratamiento	PT033							
	E. coli 37	cama tratamiento	PT034							
	E. coli 36	cama tratamiento	PT035							
	E. coli 31	cama tratamiento	PT036							
	E. coli 49	deyecciones control	PT037							
	E. coli 32	cama tratamiento	PT038							
	E. coli 33	cama tratamiento	PT039							
	E. coli 46	cama centinela	PT040							
	E. Coli 5	deyecciones tratamiento	PT041							
	E. coli 6	deyecciones tratamiento	PT042							
	S.b1	*Salmonella breundrup*								
	S. b2	*Salmonella breundrup*								
	Salmonella	*Salmonella breundrup*								

Figura 3. Dendrograma del análisis de conglomerados de los aislados de *E. coli* generado por el programa informático Gel Compar II, versión 5.10, utilizando el método de las medias aritméticas de grupos de pares no ponderados (UPGMA), con un 1% de tolerancia. Se observan los genes de resistencia detectados en cada aislado demarcado con un color. Los aislados corresponden a muestras obtenidas previo al inicio del tratamiento y el primer punto de muestreo (1 día postratamiento), a partir de los tres grupos experimentales, tratamiento, centinela (a una distancia de 30 cm) y contiguo.

De acuerdo con este análisis se observa una alta diversidad clonal de *E. coli* aislada a partir de las muestras de deyecciones y cama desde los diferentes grupos. Sin embargo, en alguno de los aislados se determinó una similitud del 100%, por lo que esos aislados son considerados clonales. Considerando este porcentaje de similitud (del 100%) se establecieron 42 patrones de PFGE (Figura 3).

Se observó que las cepas de *E. coli* aisladas previo al inicio del tratamiento presentan una similitud mayor al 95% solo con uno de los aislados obtenidos a partir de muestras de deyecciones del grupo centinela adyacente al tratamiento (grupo no tratado) y con un aislado a partir de una muestra control. Este último aislado era proveniente de una muestra de deyecciones a partir de un ave del mismo lote y eclosión, pero criada en otras condiciones y dependencias. Estos aislados obtenidos a partir del muestreo previo al tratamiento se clasificaron en el *cluster* I, donde se encuentran solamente aislados a partir de deyecciones de los distintos grupos.

Por su parte, para los aislados obtenidos después del primer día post-tratamiento, se observó que dos cepas aisladas desde deyecciones de las aves del grupo distanciado a 30 cm del tratamiento presentaron un 100% de similitud con las cepas aisladas desde deyecciones del grupo tratamiento. También, se evidenciaron cepas clonales de muestras aisladas del grupo tratado y el contiguo a este (Figura 3). En cuanto a los aislados de *E. coli* a partir de las muestras de cama, se observó una similitud del 100%, entre dos cepas del grupo tratamiento y tres cepas del grupo contiguo a este.

Los genes *tet*(A) se encontraron casi en la totalidad de los aislados, tanto previo como posterior el tratamiento, y ninguno de los aislados presentó genes *tet*(D) y *tet*(E). Además, en los aislados de los clusters IV, V, VII y VIII se observó la presencia de genes *tet*(B), *tet*(C) y *tet*(M), los cuales provenían exclusivamente desde muestras de cama. Solo cuatro aislados, uno proveniente de muestras de deyecciones y tres desde camas, eran negativos a la presencia de los genes *tet* analizados en este estudio.

DISCUSIÓN

En el presente estudio se observó que la mayoría de los aislados resistentes provenían de las deyecciones y las camas de las aves tratadas con una formulación farmacéutica de OTC. Del análisis molecular de las cepas provenientes del primer punto de muestro, se determinó que el fenotipo para la resistencia a la tetraciclina coincidía con la resistencia genotípica, la cual fue determinada mediante la positividad de genes *tet* en casi la totalidad de los aislados de *E. coli.* Solo se observó que cuatro aislados resistentes no tenían ninguno de los genes de resistencia analizados en el presente estudio, por lo que, la resistencia de estas cepas podría estar mediada por otro gen diferente.

Para tetraciclinas se han descrito diferentes genes de resistencia, donde se han nombrado genes *tet* hasta el final del alfabeto romano, e incluso se están asignando números para denominar nuevos genes *tet*. De los genes *tet* descritos, estos codifican para diferentes mecanismos de resistencia, como bombas de eflujo, protección ribosomal, modificación enzimática e incluso mecanismos desconocidos (Chopra y Roberts 2001). Por lo que, es posible que uno de los genes descritos, diferentes a los genes *tet*(A), (B), (C), (D), (E) y (M), puedan estar mediando la resistencia de los aislados que muestran resistencia fenotípica ante la prueba de difusión en disco.

Por otro lado, es posible atribuir esta resistencia a un nuevo gen no descrito. Davis et al., (2010), observaron para otra familia de antimicrobianos que, durante la caracterización de los fenotipos de resistencia, estos no eran explicados con el genotípico, y pudieron determinar que el responsable de esta condición era un nuevo gen de resistencia a los aminoglucósidos, perteneciente a la familia de genes de la metilasa del ARN ribosomal 16S denominado *rmtE.*

También, esta discrepancia entre la resistencia fenotípica y genotípica se podría deber a mutaciones de los genes de resistencia. En un estudio previo, donde se evidenció esta diferencia entre las pruebas genotípicas frente a las fenotípicas de resistencia a la rifampicina en *Mycobacterium tuberculosis*, se determinaron mutaciones en las regiones de resistencia (Rapún et al, 2019).

Considerando esto, y la discrepancia entre las pruebas convencionales de pruebas fenotípicas en comparación con las pruebas moleculares, como PCR convencional, una de las mejores alternativas para dilucidar estas diferencias es la secuenciación del genoma completo, con la finalidad de identificar una posible asociación entre el genotipo y fenotipo.

Debido a que mediante este análisis exhaustivo es posible determinar toda la secuencia de ADN del genoma de un organismo de una sola vez (Yin et al., 2019).

En cuanto a la mayor proporción de aislados resistentes, en este estudio, estos fueron determinados en muestras provenientes de las aves tratadas. Estos resultados son similares a los descritos por Berge *et al.* (2005), quienes estudiaron los patrones de resistencia bacteriana en *E. coli* de muestras fecales de ganado bovino, luego de una dosis única de florfenicol. Como resultado, ellos observaron un aumento en el número de aislados de *E. coli* resistentes. Asimismo, Fairchild *et al*. (2005) investigaron los efectos de la administración de tetraciclinas en bacterias comensales provenientes de aves comerciales, observando en el estudio de sensibilidad que *Enterococcus* spp. y *E. coli*, presentaban resistencia a tetraciclinas, y estos últimos albergaban genes *tet*(A) (32,2%) o *tet*(B) (30,5%). Este aspecto cobra relevancia ya que estas bacterias podrían ser un reservorio de genes de resistencia, lo cuales pueden ser transmitirlos a otros microorganismos (Souf *et al.,* 2011).

Nuestros resultados muestran que el gen más prevalente en los aislados resistentes fue *tet*(A), presente en el 80% de las cepas analizadas. Este hallazgo es consistente con los resultados de estudios previos, donde los genes de mayor prevalencia reportados han sido los genes *tet*(A) y *tet(*B) (Al-Bahry *et al.,* 2013; Seifi y Khoshbakht, 2016). En un estudio más actual, Sreejith *et al.* (2020), determinó a través del análisis de susceptibilidad que el 77% de los aislados del estudio *E. coli* eran resistencia a tetraciclinas, donde el 85,18% de los aislados presentaron genes *tet*(A) y el 22,22% *tet*(B). Por su parte, el segundo gen más prevalente fue *tet*(M), el cual se encontró presente en el 14% de los aislados. Otros estudios han determinado una prevalencia del *tet*(M) entre un 5% y 13% en cepas de *E. coli* (Jurado-Rabadán *et al.,* 2014; Titilawo *et al.,* 2015; Seifi y Khoshbakht, 2016). En contraste, los genes *tet*(D) y *tet*(E) no fueron detectados en ninguno de los aislados analizados en este estudio.

De acuerdo con los resultados obtenidos a partir del análisis de independencia entre la susceptibilidad de las cepas de *E. coli* y la condición de tratamiento, se observó que existe dependencia entre las dos variables. La mayor proporción de los aislados resistentes fue determinada en el grupo tratado, donde las concentraciones de OTC y su epímero eran superiores a los 22.000 $\mu g\ kg^{-1}$. Estos resultados son esperables, ya que se ha descrito que concentraciones elevadas de antimicrobianos producen una presión de selección sobre bacterias resistentes (Martínez, 2017). Sin embargo, se ha descrito que bajas concentraciones de residuos, incluso bajo la concentración mínima inhibitoria (MIC) también pueden estar relacionadas a la presencia de microorganismos resistentes (Anderson y Huges, 2014; Gullberg *et al*., 2014; Wistrand-Yuen *et al*., 2018), lo que se condice con la

detección de algunas cepas resistentes en los grupos no tratados donde, los residuos de OTC se encontraban bajo los límites de detección, es decir bajo los 12,5 μg kg^{-1}.

Este aspecto es de preocupación mundial ya que actualmente, la OTC además de ser utilizada para el tratamiento de animales productivos, es administrada en la industria avícola a través del alimento para promover el crecimiento de las aves en dosis subterapéuticas. El uso para este propósito aun es permitido en algunos países, como Brasil y China (Roth *et al*., 2019).

Sreejith *et al.,* (2020) indica que la presencia de antibióticos en los piensos y en el entorno de la granja puede ayudar a que los genes *tet*(A) y *tet*(B) persistan en el microbioma durante mucho tiempo. La presencia de antibióticos, incluso en bajas concentraciones, asegura la persistencia de estos genes resistentes, los que pueden expresarse de forma dominante en la comunidad microbiana (Sreejith *et al*., 2020). Por lo tanto, la administración de estos antimicrobianos a los animales productivos debe ser controlada, debido a que su uso puede contribuir con la selección de bacterias resistentes, las que pueden ser trasmitidas a la población humana a través de la cadena alimentaria.

En este estudio se observó que, a pesar de la alta diversidad clonal, los genes *tet*(A) son los más predominantes en las cepas analizadas, por lo que se puede afirmar que los aislados de *E. coli* resistentes se encuentran altamente representados por este gen de resistencia.

Las cepas de *E. coli*, aisladas previo al inicio del tratamiento, presentan una similitud mayor al 95% con uno de los aislados obtenidos desde el grupo centinela (adyacente al tratado) y con una muestra control proveniente de un ave criada en otras condiciones (proveniente del mismo lote de aves y grupo de eclosión). Con estos antecedentes se puede inferir que, estas cepas clonales podrían haber sido transferidas durante los primeros manejos que recibieron las aves.

Se determinó que algunos aislados resistentes de *E. coli* del grupo tratamiento y del grupo no tratado son clonales, ya que presentaron una identidad máxima del 100% en el análisis. Estos resultados nos permiten inferir que pudo ocurrir un traspaso de estos microorganismos resistentes entre los diferentes grupos. Esto podría dar respuesta a la incógnita relativa a la detección de una mayor proporción de aislados resistentes en el grupo más distanciado. En cuanto a la transferencia de microorganismos, Scott *et al.,* (2006) estudió el patrón de transmisión de *E. coli* O157:H7 entre grupos de ganado bovino, determinando la diversidad y relación de aislados en un periodo de tres meses desde las deposiciones y los corrales de los animales. Los autores determinaron que los perfiles de PFGE de todos los aislados no diferían en más de dos bandas y que se agrupaban dentro del 80% de similitud tras el

análisis del dendrograma, concluyendo que un solo aislado de *E. coli* O157:H7 se pudo transmitir a través de los corrales (Scott *et al.,* 2006). De la misma manera, Herbert *et al.,* (2014) demostraron la propagación de *E. coli* O157 entre explotaciones locales de Escocia, y destacaron en sus resultados la relación potencial entre cepas resistentes presentes en el ganado e infecciones clínicas que afectan al ser humano.

Considerando todos los resultados obtenidos en la presente investigación, podemos denotar que el uso de antimicrobianos en la producción avícola puede producir selección y persistencia de cepas resistentes, siendo un riesgo tanto para la salud humana como animal. Por consiguiente, es imprescindible monitorizar y controlar los antimicrobianos utilizados en la industria productiva animal.

CONCLUSIONES

La mayor proporción de aislados de *E. coli* no susceptibles (resistentes y de sensibilidad intermedia) a tetraciclinas se detectaron en las muestras de deyecciones y cama del grupo tratado con OTC, respecto a los grupos no tratados. Por lo que se concluye que, incluso a dosis terapéutica existe una presión de selección sobre cepas de *E. coli* resistentes a antimicrobianos de esta familia. Los genes *tet(*A) fueron los de mayor frecuencia, por lo tanto, se concluye que el principal mecanismo de resistencia de los aislados de *E. coli* a las tetraciclinas es mediado por bombas de eflujo activo. De acuerdo con el análisis de PFGE, se observó una gran diversidad clonal, aun así, se identificaron algunas cepas clonales aisladas provenientes desde deyecciones y cama del grupo tratado y no tratado, reflejando una propagación local de estos microorganismos.

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MATERIAL SUPLEMENTARIO

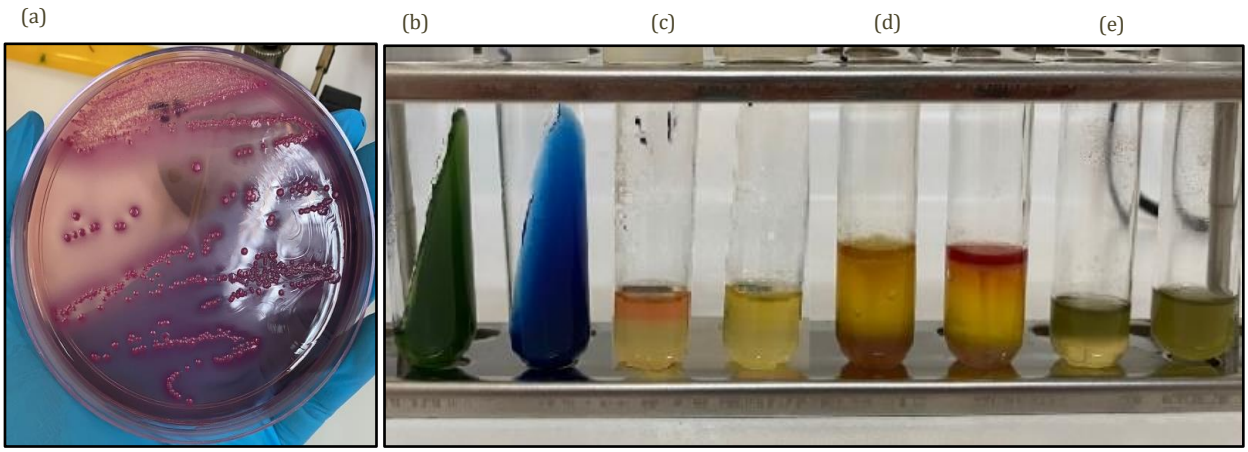

Figura suplementaria 1. (a): Muestra sembrada en agar selectivo Mc Conkey, Colonias típicas de *E. coli*: Redondas, rosas medianas; pruebas IMViC (b) Prueba de Citrato (*E. coli* negativa: coloración verde); (c) Prueba rojo de metilo (*E. coli* positiva: anillo rosado); (d): prueba de indol (*E. coli* positiva: formación de anillo); (e): Prueba Voges-Proskauer (*E. coli* negativa: color pardo).

DISCUSIÓN GENERAL

Residuos de OTC son excretados a través de las deyecciones de aves tratadas con este fármaco durante el periodo post-tratamiento, y pueden persistir en las camas por un largo periodo de tiempo. Las concentraciones cuantificadas de OTC + 4-epi-OTC en la cama de las aves tratadas fue 10 veces mayor que la encontrada en sus deyecciones al día 1 después de finalizado el tratamiento, y aun eran detectados en concentraciones de 12.946,13 µg kg^{-1} a los 21 días después de finalizado el tratamiento.

Estos resultados son concordantes con otros estudios Sarker *et al.* (2020), quienes determinaron que el 23% de las muestras de cama eran positivas a OTC, con una concentración media de 16,5 mg kg^{-1}. Otros estudios realizados en este ámbito indican que concentraciones de antimicrobianos de esta familia han sido detectados en deyecciones de pollos broiler. Yévenes *et al*. (2018) detecto 179,45 µg kg^{-1} de CTC + 4-epi-CTC incluso a los 25 días después de finalizado el tratamiento.

A pesar de que la cama de broiler recibe diferentes tratamientos antes de su uso, como por ejemplo el compostaje y digestión anaeróbicas/aeróbicas, que pueden degradar a los antimicrobianos (Ho *et al.,* 2013), es posible seguir detectando concentraciones de residuos de estos fármacos. En un estudio realizado por Feng *et al.* (2017), se describe que durante la digestión anaeróbica de estiércol de cerdo solo el antibiótico eritromicina sufrió degradación, mientras que los otros antibióticos estudiados como la claritromicina, sulfadiazina y sulfametizol fueron bastante persistentes. Previamente Dolliver *et al.* (2008), cuantificó la degradación de diferentes antibióticos en muestras fortificadas de cama de pavo durante el tratamiento de compostaje, es decir, muestras a las que se les adicionó una concentración específica del principio activo de los antibióticos a analizar. Ellos observaron que el tratamiento de compostaje puede reducir concentraciones de antibióticos en este subproducto, donde se observó que el antibiótico mayormente degradado fue clortetraciclina, seguido de monensina, tilosina y sulfametazina, siendo este último el más persistente.

Es por este motivo que la alta concentración inicial de residuos de oxitetraciclina pueden ser un riesgo, ya que pueden contribuir a la selección de bacterias resistentes a estos antimicrobianos en las camas de las aves en donde las concentraciones pueden persistir por un largo periodo. Las camas, al ser destinadas como fertilizante o insumo en las dietas de otras especies productivas, pueden contribuir en la selección de bacterias resistentes, tanto comensales como patógenas, en el tracto gastrointestinal de diferentes especies productivas. En este estudio se observó que las muestras de cama de broiler fueron las que

presentaron mayores concentraciones de OTC, debido a que en ellas se acumularon las deyecciones durante el periodo de tratamiento y postratamiento.

En cuanto a la estabilidad de los analitos durante el análisis, se determinó a lo largo del tiempo (7 y 28 días) y a diferentes temperaturas (-20ºC y -80ºC) de almacenamiento que en la matriz deyecciones el porcentaje de recuperación fue mayor al 85% ya sea en las muestras almacenadas durante 1 y 4 semana a -20ºC y -80ºC, por lo que independiente del almacenamiento de las muestras a distintas temperaturas, la recuperación se encuentra de los rangos aceptables para la cuantificación de los analitos. En el análisis de la estabilidad a los 60 días de almacenamiento a -20°C, se observó una recuperación mayor al 80% de la concentración original. Por lo que los residuos de OTC y su epímero son estables en estas matrices de estudio.

Respecto a la diseminación de residuos y bacterias resistentes a otros animales no tratados, este aspecto es de preocupación científica, debido a que Stahl et al., (2016) determinaron que cerdos no tratados que habían sido mantenidos en instalaciones previamente ocupadas por animales tratados, exhibían trazas de sulfadiazina en plasma y orina. Por su parte, en un estudio más reciente, Van de Schans *et al*. (2018) evaluó la diseminación de residuos de antimicrobianos en un criadero de aves que habían sido tratadas previamente. Ellos determinaron que la mayor contaminación de residuos fue en las áreas más expuestas al contacto directo con las aves. Sin embargo, una semana después del tratamiento la contaminación se había extendido ampliamente hacia toda la vivienda y se observó un aumento creciente de residuos de medicamentos veterinarios en los lugares más altos, como el cielo e interruptores de luz (Van de Schans *et al*., 2018). Por lo que los residuos de antimicrobianos pueden persistir por periodos prologados, por ende, ser un riesgo para el traspaso a otros animales.

Es así como, animales productivos no tratados con antimicrobianos pueden ser contaminados con estos fármacos al estar expuestos a las superficies contaminadas y/o las deposiciones con residuos provenientes de animales tratados terapéuticamente o sub-terapéuticamente, ya sea cuando las deposiciones se usan como cama de broiler para alimentación o fertilizante orgánico (Yang *et al*., 2016; Wolters *et al*., 2016; Pu et al., 2018). En el caso de las aves no se tiene conocimiento del estudio de la diseminación de residuos entre animales tratados y no tratados, bajo condiciones controladas, por lo que este estudio sería una primera aproximación sobre la diseminación de residuos y traspaso de bacterias resistentes hacia otros animales que no han sido tratados, pero que han sido criados en conjunto a animales que si han recibido tratamiento antimicrobiano.

En este estudio se observó una baja diseminación de residuos de OTC desde el grupo tratado, hacia los grupos no tratados. Debido a que solo trazas de este antimicrobiano fueron detectadas en los grupos no tratados. Esta diseminación se podría atribuir al comportamiento normal de las aves, ya que ellas realizan baños de polvo (Hartung y Schulz 2007), por lo que los residuos de OTC podrían ser movilizados junto con el polvo durante este comportamiento.

En cuanto a la excreción de OTC, en el presente estudio se determinó la presencia de este antimicrobiano durante todo el periodo post-tratamiento de las aves. Esto se observó incluso hasta el último día de muestreo, correspondiente al sacrificio de las aves, y cumplido el periodo de resguardo para la formulación farmacéutica utilizada. Las concentraciones detectadas en las deyecciones de las aves se encontraron por sobre los 700 µg kg^{-1}.

En estudios previos también ha sido posible determinar residuos de este antimicrobianos, Li et al. (2013) detectó en nueve granjas, diferentes antibióticos en las deposiciones de pollos de engorde. Dentro de los antibióticos detectados, el que presentó las concentraciones más altas fue la oxitetraciclina, y el de mayor ocurrencia fue la clortetraciclina. Asimismo, Hou *et al.* (2015) detectaron residuos de clortetraciclina en deyecciones de pollos, en donde las concentraciones máximas fueron de 2.750 mg kg^{-1}. Por su parte, Berendsen *et al.* (2018) estudiaron la persistencia de diferentes antibióticos en deyecciones de aves, para esto las muestras fueron contaminadas intencionalmente con 46 antimicrobianos y almacenadas, para el posterior análisis. Los autores pudieron modelar la disipación de los residuos y observaron que el tiempo de degradación del 90% de los antibióticos pertenecientes a las familias de las tetraciclinas se alcanzó a los 442 días.

Referente a la comparación de los niveles de estos residuos con otros tejidos comestibles y no comestibles a partir de aves tratadas, Cornejo et al. (2017), observaron que los residuos de OTC y su epímero disminuyeron rápidamente en músculo e hígado, ya que al día 3 postratamiento las concentraciones se encontraban bajo el limite de cuantificación, es decir en una concentración menor a 27 µg kg^{-1}. Por su parte en garras y plumas, residuos de estos analitos persistieron hasta 19 y 22 días postratamiento, respectivamente (Cornejo et al., 2016; Cornejo et al., 2017). Por lo que, los residuos al ser metalizados durante este periodo pueden ser eliminados a través de las deposiciones de los animales tratados. En este estudio, se evidencio que las concentraciones en las deyecciones de las aves tratadas se mantuvieron durante todo el periodo post-tratamiento, en concentraciones por sobre los 300 µg Kg^{-1}. Es así, como el uso del estiércol o desechos de los animales de abasto plantea un grave riesgo para la agricultura y la producción animal y, por ende, a la seguridad alimentaria

y la salud pública, debido a que a través de estos subproductos se puede contribuir a la contaminación ambiental con residuos de antimicrobianos (Gothwal y Shashidhar, 2014; Zhang *et al*., 2015).

En cuanto a la transmisión de cepas bacterianas, Scott *et al.*, (2006) estudiaron durante un periodo de 3 meses la diversidad y relación entre aislados de *E. coli* O157:H7 obtenidos desde aislados ambientales y fecales de bovinos, para determinar el patrón de transmisión de *E. coli* O157:H7 entre grupos de ganado. Ellos determinaron que los perfiles de PFGE de todos los aislados no diferían en más de dos bandas y se agrupaban dentro del 80% de similitud tras el análisis del dendrograma. Por lo que concluyeron que un solo aislado de *E. coli* O157:H7 puede transmitirse rápidamente a través de los corrales, siendo el entorno un reservorio para la transmisión de esta bacteria (Scott *et al.,* 2006).

De acuerdo con los resultados obtenidos en el presente estudio se acepta la hipótesis de que residuos de OTC son excretados por aves tratadas y persisten en las camas de estas, ya que se detectaron y cuantificaron residuos de este principio activo por un tiempo prolongado que llegó a abarcar los 14 días, después del sacrificio de las aves. Además, se determinaron diferencias significativas entre los grupos, considerado como factor los aislados sensibles y resistentes tanto en las deyecciones y cama. Las muestras que presentaron mayores concentraciones de residuos de OTC o niveles detectables, presentaron una mayor cantidad de aislados resistentes.

CONCLUSIONES

OTC y 4-epi-OTC fue detectado en las deyecciones y la cama de los pollos broiler tratados terapéuticamente durante todo el periodo post-tratamiento. Las aves del grupo tratado excretaron concentraciones de este fármaco, entre 347,63 µg kg^{-1} y 2244,66 µg kg^{-1}. Asimismo, se observó una mayor concentración en las camas de estas aves, donde las concentraciones oscilaron entre 10.360,60 y 22.741,68 µg kg^{-1}. En conjunto a esto, la mayor proporción de aislados de *E. coli* no susceptibles (resistentes y de sensibilidad intermedia) a tetraciclinas se detectaron en las muestras de deyecciones y cama del grupo tratado con OTC. Por lo que se concluye que, incluso a una dosis terapéutica, existe una presión de selección sobre cepas de *E. coli*, tanto en el tracto intestinal como en las camas de estas aves.

Por su parte, en los grupos centinelas, sólo se detectaron trazas de concentraciones de OTC en las deyecciones y la cama, y se determinaron aislados resistentes de *E. coli* con un 100% de similitud a partir del análisis de PFGE. Estos hallazgos podrían establecer la primera

evidencia de que hay una probabilidad, aunque sea baja, de transferencia de residuos de OTC, como de bacterias resistentes, en animales que son tratados en corrales adyacentes. En cuanto a los genes de resistencia detectados a partir de los aislados de *E. coli* resistentes a tetraciclinas, se determinó que *tet(*A) fue el gen de mayor frecuencia, por lo que se concluye que el principal mecanismo de resistencia de estos aislados de *E. coli* es mediado por bombas de eflujo activo. En el análisis de PFGE se observó clonalidad de algunas cepas detectadas a partir de muestras del grupo tratado y no tratado, lo que refleja la importancia de la propagación local de estos microorganismos.

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CAPÍTULO SUPLEMENTARIO

Determination of five antimicrobial families in droppings of therapeutically treated broiler chicken by high-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT Antimicrobials are currently used in poultry for disease treatment. However, their excretion in bird feces may contaminate the environment. Considering this, the objective of this work was to quantify antimicrobials residues concentrations in therapeutically treated broiler chicken droppings throughout the post-treatment period. For this aim a multiresidue method using high-performance liquid chromatography-tandem mass spectrometry **(HPLC-MS/MS)** was validated. Forty-eight male broiler chickens were distributed and treated with commercial formulations of 5 different antimicrobials. Results showed that oxytetracycline and 4-epi-oxytetracycline, presented the highest concentrations during all sampling period, detecting concentrations of 1471.41 μg kg^{-1} at the last sampling point (day 22 post-treatment). Florfenicol, tylosin, enrofloxacin, and ciprofloxacin were eliminated and detected in treated chicken droppings until d 18 post-treatment. Sulfachloropyridazine decrease gradually during post-treatment period until day 30. Results demonstrate that studied antimicrobials in treated chicken droppings were eliminated for prolonged periods, therefore becoming a significant route of residues dissemination into the environment.

Key words: antimicrobial, chicken droppings, therapeutically treated broiler, HPLC-MS/MS, multiresidue detection

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INTRODUCTION

In recent years, the poultry industry has expanded due to population growth and increased individual consumption, as chicken meat has a high protein content at a low price. However, this intensive production goes hand in hand with the use of antimicrobials to treat different diseases that can affect the birds during the farming process. Although, the use of these compounds is regulated in most of the countries, it must be considered that still in some large poultry producing countries such as China and Brazil; antimicrobials are still licensed for use as growth promoters (Roth et al., 2019).

Among the different families of antimicrobials used in poultry production are tetracyclines, macrolides, quinolones, phenicols, and sulfonamides (Sumano López et al., 2010). Generally, these drugs are administered to the entire flock through food or drinking water, and are applied to treat various pathologies, including intestinal infections such as colibacillosis, necrotic enteritis and other diseases generally caused by *E. coli, Salmonella* spp. or *Clostridium* spp., which represent an important concern in the industry as these generate enormous economic losses (Roth et al., 2019). However, residues of the drugs can persist in different products of animal origin. For this reason and to avoid adverse effects in the population, different international organizations have established maximum residue limits **(MRL)** to monitor the levels of these drugs in products of animal origin (FAO, 2018; Commission Regulation EU, 2010) and

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thus prevent concentrations above permissible levels from being transferred to consumers through the food chain (Love et al., 2012).

Inedible by-products are not controlled or monitored for any veterinary drug residue, as these are not intended for direct consumption. In the poultry industry, one of the main by-products obtained from the production of poultry is poultry litter, a mixture of food waste, animal waste such as feathers, litter material and mainly bird droppings (Dalolio et al., 2017; Ghirardini et al., 2020). This by-product is produced in large volumes, for example in the United States and Brazil, a production of more than 14 and 8 to 10 million tons per year has been described, respectively (Dalolio et al., 2017; Yang et al., 2019).

Poultry litter is generally used as a low-cost organic fertilizer, as it is an important source of nitrogen, phosphorus and trace elements for crop production, and has been shown to be effective in improving physical and biological fertility of crops (Bolan et al., 2010; Cheng and Jiang, 2014; Pizarro et al., 2019). In some countries it is also used as an input in the formulation of diets of other productive species, as it constitutes a good source of protein, energy and minerals (Cornejo et al., 2019) especially for cattle, which due to its digestive characteristics, can make a more efficient use of nutrients (Cabrera-Núñez et al., 2018). However, in the absence of any control of antimicrobial presence, these by-products can contribute to the re-entry of antimicrobial residues through the food chain, and their transfer and dissemination to the environment. The application of chicken manure containing antibiotics is considered to be one of the major sources of contamination and transfer of these compounds to the environment (Muhammad et al., 2020). This is due to the fact that once antibiotics are administered, they can be excreted in large quantities through urine and feces, and it has been reported that between 17 and 90% of them are excreted in their nonmetabolized form or as active metabolites (Massé et al., 2014).

This issue has gained relevance in recent years and different studies have determined the presence of antimicrobial residues in the manure of productive animals and poultry droppings (Berendsen et al., 2015; Yévenes et al., 2018). It has also been described that antimicrobials and their metabolites are strongly adsorbed in feces due to chemical interaction with metals and organic substances, forming complexes with soluble organic compounds that remain stable during storage (Massé et al., 2014). However, the probability of entry of these drugs to environmental reservoirs differs according to the compound and the animal species that excretes it (Spielmeyer, 2018; Jansen et al., 2019).

The application of manure as fertilizer on soils constitutes a massive entry route for these residues to the environment, as a significant fraction becomes mobile with water, polluting the surrounding environment and surface and groundwater through runoff and leaching processes, thus becoming important reservoirs of antibiotics (Tong et al., 2011; Massé et al., 2014; Slana et al., 2014; Albero et al., 2018; Conde et al., 2018).

Depending on the physicochemical properties of antibiotics, soil characteristics and environmental conditions, antibiotics can be retained in the soil or uptake by vegetables and even toward the fruits (Wang et al., 2006; Wang and Yates, 2008; Kang et al., 2013; Pan and Chu et al., 2017). Likewise, in the aquatic environment, various manifestations of toxicity have been reported in different aquatic species (Isidori et al., 2005; Park and Choi, 2008; Daghrir and Drogui, 2013; Ortiz et al., 2014; de Vasconcelos et al., 2017)

For this reason, it is important to determine the elimination of antimicrobial residues in therapeutically treated bird droppings and thus assess whether this by-product is a potential route of transfer and dissemination of antibiotics to the environment. In addition, the use of noninvasive matrices can be a useful tool for monitoring the use of antimicrobials on farms (Nebot et al., 2012).

Different studies have demonstrated the presence of antimicrobial residues in poultry feces (Slana et al., 2017; Berendsen et al., 2018; Cornejo et al., 2018; Yévenes et al., 2018). However, we are not aware of any study that quantify and project the concentrations that may persist in this matrix after the antimicrobial treatment is applied to these animals during the farming process. Therefore, the objective of this work was to quantify the concentrations of the most widely used antimicrobials in the poultry industry, by simultaneously detecting them in therapeutically treated broiler chicken droppings during the post-treatment period. For this and in order to analyze the persistence of antimicrobial of tetracycline, macrolide, fluoroquinolones, phenicols and sulfonamides families, a multiresidue method by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was validated.

MATERIAL AND METHODS

Certified Standards, Reagents, and Solvents

Analysis and quantification of analytes in droppings was performed using certified standards with a purity greater than 90%. Tetracycline **(TC)** hydrochloride, 4-epi-tetracycline **(4-epi-TC)** hydrochloride, oxytetracycline **(OTC)** hydrochloride, 4-epi-oxytetracycline **(4-epi-OTC)**, chlortetracycline **(CTC)** hydrochloride, 4-epi-chlortetracycline **(4-epi-CTC)** hydrochloride, tylosin **(TYL)** tartrate, enrofloxacin **(EFX)**, ciprofloxacin **(CFX)** hydrochloride, flumequine **(FLU)**, florfenicol **(FF)**, sulfachloropyridazine **(SCP),** and sulfadiazine **(SDZ)**, were used for the HPLC-MS/MS analysis. All standards were manufactured by Toronto Research Chemicals (Toronto, Canada), Dr. Ehrenstorfer Gmbh (Augsburg, Germany) and Sigma Aldrich (Merck KgaA, Darmstadt, Germany).

The following certified reagents were used as internal standards: isotopic tetracycline d-6 **(TC-D6)**, certified purity 80%, manufactured by Toronto Research Chemicals (Toronto, Canada); enrofloxacin D5 hydrochloride **(EFX-D5)**, certified purity 94%, manufactured by Dr. Ehrenstorfer Gmbh (Augsburg, Germany); sulfamethazine-phenyl-13C6 hemihydrate **(SMZ13C6)**, certified purity 99.8%; chloramphenicol D5 **(CAF-D5)**, certified purity 98.95% and erythromycin-N-methyl-13C, d3 **(ETM13C-D3)**, certified purity 98%, manufactured by Sigma Aldrich (Merck KgaA, Darmstadt, Germany).

A stock solution was prepared for each of the analytes of interest and internal standards at a concentration of 1,000 μg mL^{-1} in methanol. Two intermediate or working solutions at 1,000 ng mL^{-1} in methanol were prepared from the stock solutions, which contained the mix of analytes and the internal standards, respectively, and were used for spiking samples.

Solvents used for the analysis were water, methanol and acetonitrile, from the LiChroslv line, liquid chromatography grade (Merck KgaA, Darmstadt, Germany). EDTA-McIlvaine buffer was prepared with citric acid monohydrate, disodium hydrogen phosphate dihydrate and ethylenedinitrilotretraacetic acid (EDTA) disodium salt, manufactured by Merck KgaA (Darmstadt, Germany).

Extraction of Antimicrobial Residues from Chicken Droppings

The procedure was based on an analytical methodology previously published by Berendsen et al. (2015) and was optimized to detect and quantify multiple antimicrobials in animal waste by means of high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) after cleaning by solid phase extraction.

To extract the different analytes from the droppings matrix, samples were homogenized and placed in 50 mL polypropylene tubes (1 ± 0.01 g). As solvent for extraction, 8 mL of EDTA-McIlvaine buffer (pH 4.0 ± 0.1) and 2 mL of acetonitrile were used. Samples were shaken in a Multi Reax agitator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 10 min, subsequently centrifuged in an Eppendorf Centrifuge 5804 centrifuge (Merck KGaA, Darmstadt, Germany) at 3,234 g for 10 min, and filtered through glass microfiber filters without binding agents, grade GF/A (1.6 μm) (MERCK KGaA, Darmstadt, Germany). For the clean-up, SUPEL-SELECT HLB 200 mg/6 mL solid phase extraction columns (Waters Corp., Milford, MA) were used, which were previously conditioned with 5 mL of methanol and 5 mL of water, both LC-MS grade.

Then, columns were washed with 5 mL of LC-MS grade water and dried with a manifold pump for later elution with 10 mL of LC-MS grade methanol. Finally, the eluate was dried under a gentle nitrogen flow in a water bath at 40−50°C, in an automated solvent evaporation system (TurboVap LV, Biotage, Uppsala, Sweden). The samples were reconstituted with 200 μL of methanol and 300 μL of LC grade water, shacked and sonicated for 5 min, then centrifuged at 17,136 g for 5 min. Finally, the supernatant was filtered through a 0.22 μm Millex syringe filters (MERCK KGaA, Darmstadt, Germany).

Instrumental Analysis

Samples were analyzed using an Agilent 1290 series Liquid Chromatograph (Santa Clara, CA) coupled to a triple quadrupole tandem mass spectrometer, in multiple reaction monitoring mode via an electrospray interface. In particular, an AB Sciex API 5500 mass spectrometer (AB Sciex LLC, Framingham, MA) was used. This device through polarization switching was operated in positive ionization mode for the analytes TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC, TYL, EFX, CFX, FLU, SCP, SDZ, SMZ13C6, EFX-D5, ETM13C-D3, TC-D6 and in negative ionization mode for the FF and CAF-D5 analytes. The specifications of the mass spectrometer and the liquid chromatograph are described in Supplementary Table 1 while Supplementary Table 2 shows the specific mass spectrometer conditions for the analytes.

Analytical Methodology Validation

In order for this methodology to be valid for the determination of antimicrobial residues from broiler chicken droppings, and that the results obtained from experimental samples are reliable and reproducible; a validation of the analytical methodology was carried out according to an internal validation protocol, which was developed following the recommendations from Commission Decision 2002/657/EC (Commission Decision, 2002) and the guideline VICH topic GL49 (EMA, 2015). In accordance with this internal validation protocol, following parameters were evaluated to demonstrate that the analytical method reliably and accurately met the criteria applicable to performance characteristics.

Specificity The specificity of the method was determined by analyzing 21 samples from different sources, in order to determine the presence of interferents in the retention time of the analytes of interest.

Detection Range The limit of detection **(LOD)** and limit of quantification **(LOQ)** were determined in 2 steps; first, a preliminary estimation of LOD and LOQ was performed to verify the existence of a linear relationship between concentration and instrument response. These values were determined as instrumental LOD and LOQ. Subsequently, the LOD and LOQ for each analyte were determined in a fortified matrix. The criteria for establishing the LOD were to achieve a signal-to-noise ratio greater than 3:1; while, for determining the LOQ, a signal-to-noise ratio higher than 10:1.

Linearity of Calibration Curves To determine the linear response of the matrix calibration curves, we

performed a linear regression analysis of the area ratio and target levels. For this, an analysis was performed on different days of 3 calibration curves in samples free of antibiotic residues, spiked at different levels including the zero; the concentration corresponded at 12.5, 25, 50, 75, and 100 μg kg^{-1}.

Recovery and Precision Recovery was determined by analyzing blank spiked samples at 25, 50, and 75 μg kg^{-1}. The concentration present in each sample was determined after each analysis, and the resulting concentrations were used to calculate the recovery percentage using the following equation:

$$Recovery(\%) = (quantifiedconcentration * 100) / spikedlevel.$$

Precision was evaluated by analysis of repeatability and intralaboratory reproducibility. For the determination of repeatability, independent tests were carried out by the same operator using the same method, same solvent and test equipment in the same laboratory. To determine the proximity of the agreement of the results from these independent samples analyzed under the same conditions, we worked with 18 blank samples that were spiked at 3 different concentrations (0.5, 1 and 1.5 times the established limit of 50 μg kg^{-1}), with 6 replicates for each concentration.

On the other hand, intralaboratory reproducibility was determined by the same analysis, using the same working concentrations and number of replicates. However, the analyzes were performed under different laboratory conditions (different reagent lots, ambient temperatures, days, and operators).

The precision of the method was determined by calculating the relative standard deviation **(RSD)** of the concentrations detected for each spike level, in the intralaboratory repeatability and reproducibility tests.

The following analytes were included in the internal validation plan: TC, 4-epi-TC, OTC, 4-epi-OTC, CTC, 4-epi-CTC, TYL, EFX, CFX, FLU, FF, SCP and SDZ). On the other hand, the following standards labeled with stable isotopes corresponding to the nuclear component were used as internal standards: CAF-D5, EFX-D5, ETM13C-D3, SMZ13C6 and TC-D6.

Experimental Animals

The depletion study was performed with Ross 308 genetics male broiler chickens (Ross, Aviagen Inc., Huntsville, AL). These commercial hybrids have a good growth rate, good feed conversion, yield and robustness, where at 42 d of life, present a body live weight of approximately 2.9 kg with an average daily gain of 100 g (Ross, 2021), which is why they were chosen for this study.

The birds were raised from their first day of life in conditioned pens and under controlled environmental conditions of temperature (25 ± 5°C) and humidity (50 −60%), according to their life stage requirements (Ross, 2018). Throughout the experiment the birds had free access to water and food. The latter was formulated according to the nutritional requirements of the birds.

For the maintenance of the birds, the recommendations of the national animal welfare regulations of Law No. 20,380 "On Protection of Animals" (MINISTERIO, 2009) and of Directive 2010/63/EU related to the protection of animals used for scientific purposes (European Parliament and the Council of the European Union, 2018) were followed. For the slaughter of the birds, Regulation (EC) No. 1099/2009 on the protection of animals at the time of slaughter was respected (European Commission, 2009). Furthermore, the study was approved by the Institutional Committee for the Care and Use of Animals (**CICUA**, by its Spanish acronym) of the University of Chile (Certificate No. 19276-VET-UCH). All the biosecurity measures, the work with the birds and the analysis of the experimental samples, were carried out with the approval of the Biosecurity Committee of the Faculty of Veterinary and Livestock Sciences (**FAVET**, by its Spanish acronym) of the University of Chile (Certificate No. 145).

Pharmaceutical Formulations for In vivo Study

Five pharmaceutical formulations, which representing different families of antimicrobials, were used to treat the experimental animals. Specifically, for the study, oral formulations of 10% OTC, 10% TYL, 20% EFX, 2% FF, and 10% SCP were used. The products had different established withdrawal period for muscle of 7 d for OTC, 5 d for TYL, 10 d for EFX and FF, and 30 d for SCP. All these formulations are authorized for use in broiler chickens, which are registered and authorized at the national level by the Agricultural and Livestock Service (SAG by its Spanish acronym) (SAG, 2020).

Treatment and Sample Collection

Forty-eight birds were distributed into 6 groups, so that each experimental group consisted of 8 birds. The number of individuals was determined according to the recommendations of the VICH topic GL48 (EMA, 2015).

Groups A, B, C, D and E corresponded to the groups of birds that were treated with OTC, TYL, EFX, FF and SCP, respectively. The treatment was carried out orally using a No. 6 Levin gastric tube, to ensure the complete intake of the therapeutic dose of each antimicrobial and thus reduce variability due to consumption. Group A was treated with 10% OTC, with a dose of 80 mg kg^{-1} every 24 h for 10 consecutive d. Group B was treated with 10% TYL, with a dose of 35 mg kg^{-1} every 24 h for 7 consecutive d. Group C was treated with 20% EFX, with a dose of 10 mg kg^{-1} every 24 h for 7 consecutive d. Group D was treated with 2% FF, with a dose of 15 mg kg^{-1} every 12 h for 4 consecutive d, and group E was treated with 10% SCP, with a dose of 30 mg kg^{-1} every 24 h for 5 consecutive d.

On the other hand, the sixth group, called group F, corresponded to the control group and consisted of 8 birds kept under the same conditions, but without antimicrobial treatment.

After treatment, each experimental group was sampled. The samples were obtained on d 5, 7, 10, 14, 18, and 22 post-treatment. For SCP analysis, 2 additional samples were taken on d 30 and 34 post-treatment, as the withdrawal period of this pharmaceutical formulation was much longer than the other antimicrobials analyzed.

Cloaca samples were collected individually with a torula, homogenized and stored at -20°C in sterile 50 mL polypropylene tubes for further processing, extraction and chromatographic analysis.

Quantification of Residues from Experimental Samples

After analysis and detection of the samples through the multiresidue method, the concentrations of OTC, 4-epi-OTC, TYL, EFX, CFX, FF and SCP were determined using the line equation obtained from the regression analysis of the calibration curves of spiked samples, carried out together with each sampling. The samples used to construct the curves were free of residues and were spiked at different and equidistant concentrations to avoid extrapolations for the quantification of the different analytes. For the quantification, only those curves that presented a coefficient of determination R^2 greater than 0.98 were considered.

Statistical Analysis and Depletion Study

To determine whether there were differences between antimicrobials and days post-treatment, an analysis of variance (ANOVA) and Fisher's least significant difference **(LSD)** test were performed. Antimicrobial concentrations in droppings were expressed as natural logarithm **(LN)** and corresponded to the dependent variable. The days post-treatment **(DPT)**, antimicrobial **(AB)** and the interaction between both were also considered as factors. The statistical software Infostat version 2020I was used. In all experiments, differences were considered statistically significant when the associated probability level (*P-value*) was less than 0.05.

In addition, and to determine and extrapolate the depletion of residues, a regression analysis was performed with the concentrations detected vs. the time of analysis, following the recommendations of the Guidance: Approach harmonization of withdrawal periods and Guideline on determination of withdrawal periods for edible tissues (EMEA, 1996; EMA, 2018). According to these guidelines the depletion of the residues was determined with a one-sided upper tolerance limit with a 95% of confidence according to Stange equation.

RESULTS

Multiresidue Methodology Validation

In the specificity analyzes, no interferents were observed in the retention times for each analyte studied from the samples of chicken droppings free of antimicrobial residues. The retention times for each analyte are described in

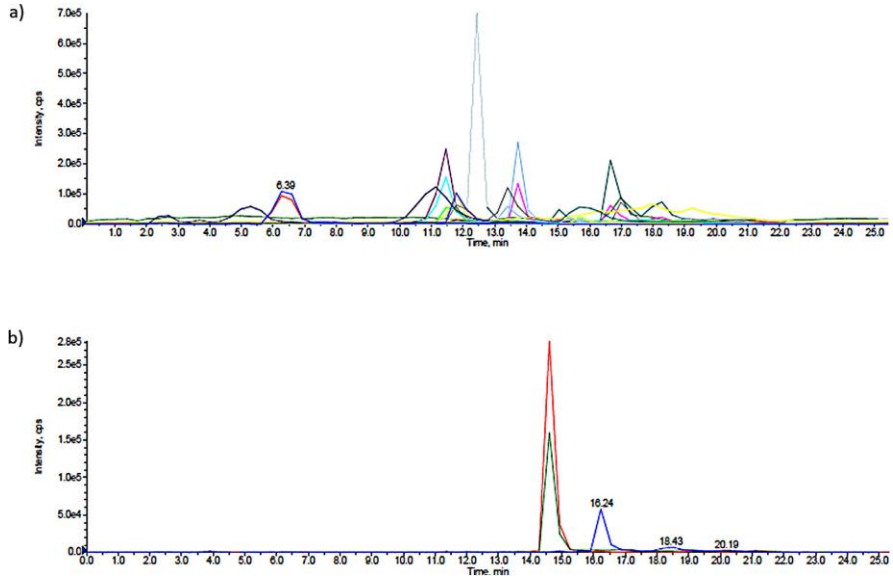

Figure 1. (A) Representative chromatogram of droppings blank sample spiked with 50 μg kg^{-1}, in positive mode. (B) Representative chromatogram of droppings blank sample spiked with 50 μg kg^{-1}, in negative mode.

Table 1. Limit of detection and limit of quantification for each studied analyte.

Analyte	IDL[1] (μg kg^{-1})	IQL[2] (μg kg^{-1})	LOD[3] (μg kg^{-1})	LOQ[4] (μg kg^{-1})
Florfenicol	3.3	11.1	11.2	33.5
Tylosin	3.4	11.4	7.3	21.9
Enrofloxacin	2.0	6.7	10.7	32.0
Ciprofloxacin	3.1	10.4	5.8	17.5
Flumequine	2.9	9.7	11.7	35.1
Sulfachloropyridazine	3.0	10.1	7.4	22.2
Sulfadiazine	2.8	9.5	12.3	36.8
Tetracycline	3.4	11.4	11.9	35.8
4-epi-tetracycline	2.9	9.7	12.0	36.0
Oxytetracycline	2.7	8.9	12.1	36.4
4-epi-oxytetracycline	2.3	7.8	12.2	36.5
Chlortetracycline	3.3	11.1	12.5	37.4
4-epi-chlortetracycline	2.9	9.6	11.7	35.1

[1]Instrumental Limit of detection.
[2]Instrumental Limit of quantification.
[3]Limit of detection in matrix.
[4]Limit of quantification in matrix.

Supplementary Table 3. Supplementary Figures 1 and 2 show the chromatograms of the analyzed analytes from the injection of certified purity standards, using the conditions established for the analytical method in the API 5500 spectrometer (ABSciex). Figure 1 show a representative chromatogram (positive and negative mode, respectively) of a sample spiked with 50 μg kg^{-1}.

The instrumental and spiked matrix LOD and LOQ for each analyte are shown in Table 1. LODs for the analytes in the droppings matrix were equal to or less than 12.5 μg kg^{-1} (Table 1). The LOQ presented values between 17.5 and 37.4 μg kg^{-1} (Table 1). From these concentrations, experimental samples could be reliably and accurately quantified.

The curves analyzed presented a linear response in the area ratio vs. concentration regression analysis, with an R^2 greater than 0.99 for all the analytes of interest, as shown in Table 2.

The recovery of the analytes met the minimum acceptance criteria according to the internal validation protocol, where the recovery percentage for the working concentrations used had to be between 90 and 110%. The recovery ranges obtained in the study ranged from 91.9% to 108.1%, with the lowest and highest recovery value for CFX. Regarding the precision of the method, the results for the repeatability test presented a lower RSD than the results obtained for intralaboratory reproducibility, and these values did not exceed 23% of variation. The intralaboratory repeatability and reproducibility values are shown in Table 2.

Quantification of Antimicrobials in Droppings

The quantified concentrations for the different antimicrobials in the droppings of therapeutically treated birds are shown in Table 3.

A tendency to increase in concentration was observed for OTC and its epimer throughout the post-treatment period. For this antimicrobial, the value of the last sampling point was approximately 69% higher than the concentrations detected in the first sampling point, corresponding to d 5 post-treatment. This coincides with the pharmacokinetic characteristics of OTC, which presents an accumulation in adipose tissue due to its great fat solubility (Vicente and Pérez-Trallero, 2010).

A similar behavior was observed for TYL, EFX and CFX. However, the concentrations detected were much lower than those of OTC and its epimer, and the highest standard deviation for these analytes was observed in the fifth sampling point, corresponding to 22 d post-treatment (Table 3 and Figure 2).

The concentrations detected for FF remained constant during the analysis period and although an increase in concentrations was observed with the passage of days after treatment, this increase was more marked in the second sampling point, where 274.07 μg kg^{-1} were detected (vs. 134.70 μg kg^{-1} detected on the first sampling point).

On the other hand, a decrease in the elimination of SCP was observed throughout the samplings. In the first sampling point, 516.61 μg kg^{-1} were quantified in the matrix, and on the penultimate sampling point (d 30 post-treatment, corresponding to the withdrawal period established for the pharmaceutical formulation for muscle), 34.04 μg kg^{-1} were detected and quantified, which is equivalent to a 93.4% decrease in residues. On the last sampling point, corresponding to d 34 post-treatment, it was only possible to detect traces of this antimicrobial, which were found below the LOD of the analytical methodology.

According to the results obtained from the ANOVA model, the AB factor and the AB-DPT interaction were significant ($P\text{-value} < 0.05$), but the DPT effect was not. The model explained 73% of the variation observed in LN. In the Fisher's LSD, there were significant differences for the AB factor between all the means for each antibiotic used, with TYL being the antibiotic with the lowest mean and OTC and its epimer the antibiotic with the highest mean. Furthermore, significant differences were observed between all means according to the interaction between the DPT and AB factors, the lowest mean being for TYL at 14 d post-treatment and the highest mean for OTC and its epimer at 14 d post-treatment.

Projection of Antimicrobials Persistence in Broiler Chicken Droppings

The results were graphed on a semilogarithmic scale of concentration vs. time and a linear regression analysis was performed considering a confidence level of 95%. Data that were below the LOQ of the analytical methodology were set to one-half of the LOQ.

For the SCP, TYL, EFX and CFX, a projection was made and through this analysis we determined the time in which the concentrations would be equal to or lower than the 50 μg kg^{-1} established limit and the LOQ

Table 2. Validation parameters of analytical methodology for antibiotic detection from chicken droppings.

Analyte	Spike level (μg kg^{-1})	Recovery[a] (%)	RSD$_r$[b] (%)	RSD$_{RL}$[c] (%)	Linearity[d] ($R^2 \pm$ SD)
Florfenicol	25	97.5	3.7	13.0	0.996 ± 0.002
	50	102.5	3.9	12.4	
	75	99.2	1.2	4.3	
Tylosin	25	106.2	3.6	12.8	0.995 ± 0.003
	50	93.8	3.8	14.5	
	75	102.1	1.2	4.5	
Enrofloxacin	25	97.4	2.2	12.2	0.996 ± 0.005
	50	102.6	2.1	11.6	
	75	99.1	0.7	4.0	
Ciprofloxacin	25	91.9	3.1	10.1	0.996 ± 0.003
	50	108.1	2.8	8.6	
	75	97.3	1.0	3.2	
Flumequine	25	97.6	2.0	21.5	0.996 ± 0.004
	50	102.4	2.1	20.5	
	75	99.2	0.7	7.1	
Sulfachloropyridazine	25	97.3	3.7	9.7	0.999 ± 0.001
	50	102.7	3.5	9.2	
	75	99.1	1.2	3.2	
Sulfadiazine	25	94.0	4.9	10.4	0.996 ± 0.004
	50	106.0	4.8	9.3	
	75	98.0	1.6	3.3	
Tetracycline	25	100.4	3.9	7.0	0.997 ± 0.002
	50	99.6	3.9	7.1	
	75	100.1	1.3	2.3	
4-epi-tetracycline	25	99.7	4.0	21.2	0.999 ± 0.000
	50	100.3	4.1	21.1	
	75	99.9	1.3	7.1	
Analyte	Spike level (μg kg^{-1})	Recovery[1] (%)	RSD$_r$[2] (%)	RSD$_{RL}$[3] (%)	Linearity[4] ($R^2 \pm$ SD)
Oxytetracycline	25	104.3	3.3	4.7	0.995 ± 0.004
	50	95.7	3.2	5.2	
	75	101.4	1.1	1.6	
4-epi-oxytetracycline	25	98.4	3.3	21.3	0.997 ± 0.003
	50	101.6	3.2	20.6	
	75	99.5	1.1	7.0	
Chlortetracycline	25	100.8	2.7	14.8	0.995 ± 0.003
	50	99.2	2.8	15.1	
	75	100.3	0.9	5.0	
4-epi-chlortetracycline	25	103.6	4.2	7.9	0.996 ± 0.002
	50	96.4	4.4	8.5	
	75	101.2	1.4	2.7	

[1]Recovery percentage (%) from spiked matrix.
[2]Relative Standard Deviation of repeatability.
[3]Relative Standard Deviation of intralaboratory reproducibility.
[4]Linearity of 3 calibration curves in matrix spiked at 12.5, 25, 50 and 100 μg kg^{-1} (R^2: coefficient of determination ± Standard Deviation).

established for each analyte, with 95% confidence. As the values obtained were a fraction of a day, the depletion period was considered as the value rounded to the next unit.

The projected days for the depletion of SCP were 42.70 and 49.91, considering the cut-off point established in the study (50 μg kg^{-1}) and the LOQ of the analytical methodology (22.2 μg kg^{-1}), respectively, with 95% confidence (Figure 3).

For EFX and its active metabolite, the projected days for depletion were 49.44 and 55.76, considering as cut-off point the established limit and the LOQ of the analytical methodology, respectively, with 95% confidence (Figure 4).

The days projected for the depletion of TYL were 100.78 and 131.51, considering as cut-off point the established limit and the LOQ of the analytical methodology, respectively, with 95% confidence (Figure 5).

On the other hand, for OTC and its epimer, a projection was not possible because the concentrations tended to increase after treatment. In the case of FF, the projection could not be carried out either because its concentrations remained above 100 μg kg^{-1} during the first 4 sampling points in little variable ranges and only at the fifth sampling point, corresponding to 22 d post-treatment, the quantified concentrations were under the LOD (11.2 μg kg^{-1}) of the analytical methodology. Therefore, when projecting the results by linear regression, the depletion period of FF residues was overestimated.

DISCUSSION

Poultry droppings are the main component of the poultry litter. This by-product is produced in high volumes and is used to feed other productive species or as an organic fertilizer (Slana et al., 2014). The results of this study show that antibiotic residues remain in droppings for long periods and their excretion can even increase during the post-treatment period. This

Table 3. Average concentration (μg kg^{-1}) of oxytetracycline plus 4-epi-oxytetracycline, enrofloxacin plus ciprofloxacin, tylosin, florfenicol and sulfachloropyridazine at different d post-treatment in droppings of treated broiler chickens.

			Average concentration (μg kg^{-1})				
Sampling point	Post-treatment day	Day of life of the birds	OTC + 4-epi-OTC[1]	EFX + CFX[2]	TYL[3]	FF[4]	SCP[5]
1	5	25	872.04	113.54	104.66	134.70	516.61
2	10	30	754.15	68.23	66.50	274.07	141.56
3	14	34	2058.97	283.78	37.70	126.43	171.17
4	18	38	1481.77	75.69	71.84	156.55	319.84
5	22	42	1471.41	<LOQ[6]	ND[7]	<LOD[8]	20.99
6	30	46	-	-	-	-	34.04
7	34	50	-	-	-	-	<LOD[9]

[1]Oxytetracycline plus 4-epi-oxytetracycline.
[2]Enrofloxacin plus ciprofloxacin.
[3]Tylosin.
[4]Florfenicol.
[5]Sulfachloropyridazine.
[6]Enrofloxacin: 32 μg kg^{-1}, only one sample quantified above the Limit of quantification of 38.02 μg kg^{-1} for enrofloxacin and 20.98 μg kg^{-1} for ciprofloxacin).
[7]Non detected.
[8]Limit of detection in matrix for florfenicol: 11.2 μg kg^{-1}.
[9]Limit of detection in matrix for sulfachloropyridazine: 7.4 μg kg^{-1}.

coincides with the research of Spielmeyer (2018), who indicated that antibiotic residues were present in the excreta of different animals including poultry, and that the percentage of the active ingredient ranged from <5% to 90% depending on the substance used days after the end of treatment, which did not correlate with the concentrations of residues that could be detected in edible tissues.

Also, multiresidue methods have gained relevance in recent years because they allow the simultaneous detection of different analytes from the same sample, being very useful for detecting residues of importance for public and animal health (Nebot et al., 2012). Currently, analytical methods using HPLC-MS/MS for the detection of antimicrobial residues from animal waste have been developed by different authors (Jansen et al., 2019; Bajkacz, et al., 2020; Patyra et al., 2020;Zheng et al., 2021). However, in the present study, a multiresidue analytical methodology based on the one published by Berendsen et al., (2015) was optimized and validated for the detection of different analytes in droppings of broiler chickens.

Through the use of the validated multiresidue method, this study showed that antibiotic residues belonging to the families of tetracyclines, sulfonamides, quinolones, macrolides and phenicols, persist in the

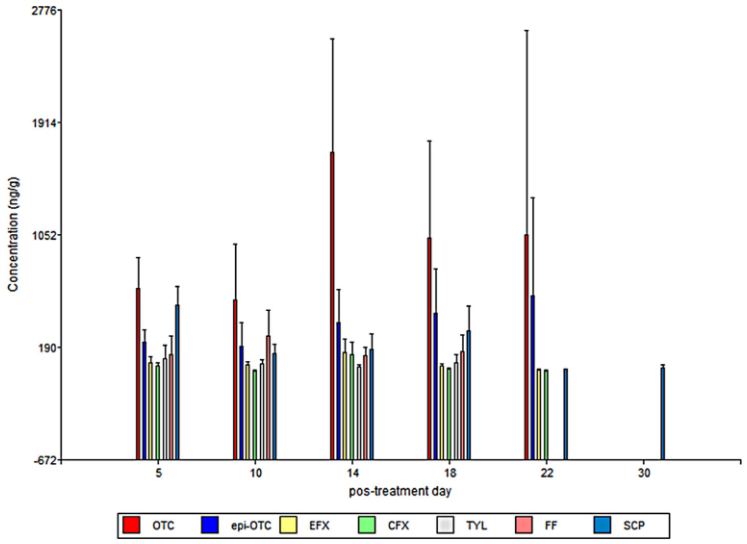

Figure 2. Concentrations of oxytetracycline, 4-epi-oxytetracycline, enrofloxacin, ciprofloxacin, florfenicol, tylosin and sulfachloropyridazine detected in broiler chicken droppings during the post-treatment period, after the administration of antimicrobials in therapeutic doses. Error bars represent the standard deviation.

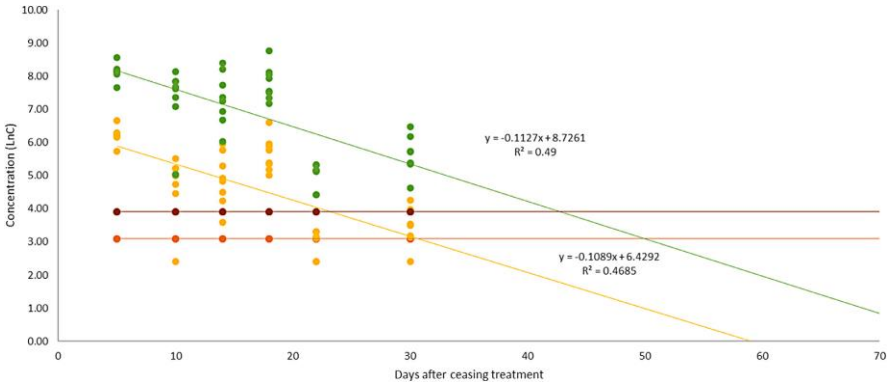

Figure 3. Projection of sulfachloropyridazine concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for sulfachloropyridazine (22.2 μg kg^{-1}); dark red: established limit of 50 μg kg^{-1}.

droppings of antibiotic-treated birds for periods longer than withdrawal times and even longer beyond the slaughter of the birds. They can therefore persist in the litter or manure and spread to the environment or to other animals as part of the diet. Therefore, poultry litter represents a risk and an unknown route for the reentry of antibiotic residues through the food chain and their transfer to the environment (Slana et al., 2017). It is important to consider that the persistence of these drugs varies according to the species, Berendsen et al. (2018) evaluated the persistence of a wide range of antibiotics during the storage of calf, pig and broiler chicken droppings, and found that tetracyclines were moderately persistent or very persistent in calf and broiler chicken manure and slightly less persistent in pig manure. In contrast, sulfonamides showed a high withdrawal rate in most manure samples, with a half-life between 0.2 and 30 d overall (Berendsen et al., 2018).

Previous studies have analyzed antimicrobial depletion in broiler chicken droppings (Cornejo et al., 2018; Yévenes et al., 2018). Yévenes et al. (2018) indicate that residues of FF, SCP, CTC and 4-epi-CTC persist in broiler chicken droppings and could be a risk to public health. The authors reported that the longest excretion period was obtained for CTC, a tetracycline, for which the highest concentrations were also detected after the end of the treatment with therapeutic doses. In the present study, the highest concentrations were also observed for an antibiotic of the tetracycline family, OTC and its epimer, which even exceeded the concentrations of the other antimicrobials by more than 10 times at the same post-treatment day. Additionally, an increase of excretion of OTC and its epimer was observed throughout

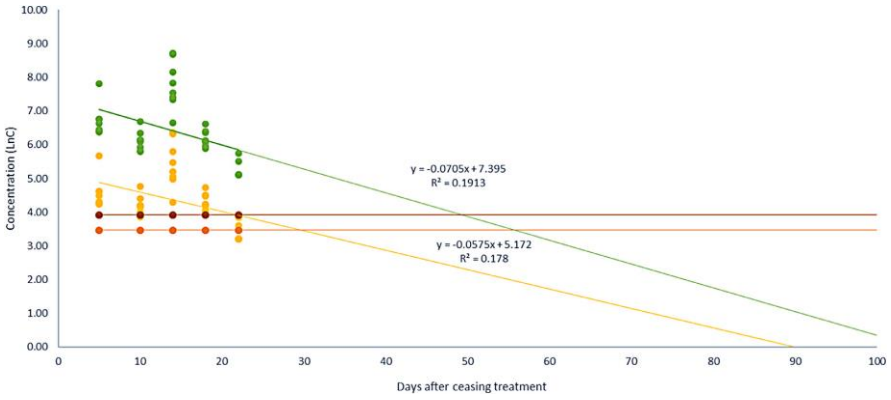

Figure 4. Projection of enrofloxacin and its metabolite concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for enrofloxacin (32 μg kg^{-1}); dark red: established limit of 50 μg kg^{-1}.

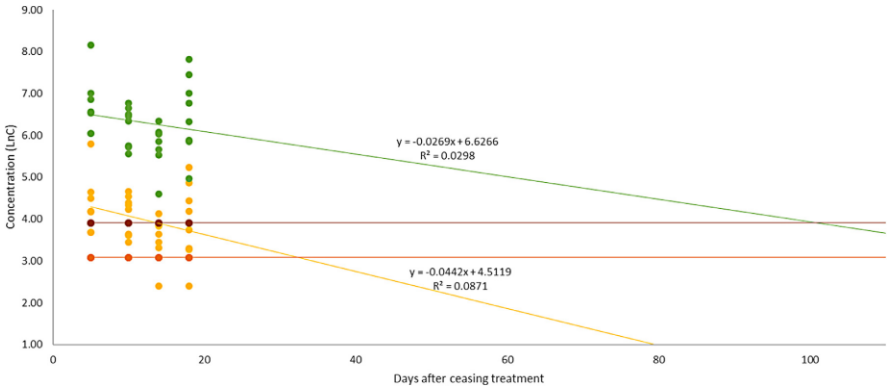

Figure 5. Projection of tylosin concentrations detected in broiler chicken droppings during the post-treatment period. Green: concentrations in LN considering 95% confidence; yellow: concentrations in LN; orange tree: LOQ of the analytical methodology for tylosin (21.9 μg kg^{-1}); dark red: established limit of 50 μg kg^{-1}.

the samplings, which may be due to the fact that these antimicrobials are absorbed quickly and completely in the gastrointestinal tract, presenting a variable binding to plasma proteins. In addition, they are distributed throughout all tissues and have a high affinity for bone tissue. They are partially metabolized throughout the body, being eliminated through urine and feces (Vicente and Pérez-Trallero, 2010). These characteristics could explain why the concentrations detected would not correlate with those of edible tissues measured in other studies, in which a depletion of residues is observed in muscle, liver and even feathers (Cornejo et al., 2017).

Detected concentrations of FF were higher than 100 μg kg^{-1} on almost all samplings, and only on the last sampling point (d 22 post-treatment) were traces found, below the LOD. In previous studies by Yévenes et al. (2018) the concentrations of this antibiotic decreased more rapidly, being below the LOD (50 μg kg^{-1}) on d 15 post-treatment.

For TYL, an alternation of decrease and increase of concentrations was observed. TYL absorption is relatively poor orally (Gutiérrez et al., 2018), which would explain why high concentrations were detected on the first sampling point (d 5 post-treatment). Then, there was a decreasing trend in concentration on the second and third sampling points and then a slight increase on the fourth sampling point. However, at 22 d post-treatment, it was only possible to detect TYL residues in one of the samples below the LOQ (32 μg kg^{-1}), which was registered as not detected. Fluctuations in TYL concentrations during d 14 and 18 posttreatment (Table 3) could be related with the metabolism and recirculation of this antimicrobial from other tissues of the bird, such as liver or fat, because like OTC, TYL is a lipophilic antibiotic (Ozdemir et al., 2018).

EFX and its metabolite CFX showed a marked increase on the third sampling point (d 14 post-treatment), increasing to more than double the concentrations obtained on the first sampling point (d 5 post-treatment). Unlike our results, Slana et al. (2014) studied the EFX pattern and indicated that after the end of the treatment, from d 8 onward, no metabolites were observed in the excreta; however, EFX was continuously excreted until the end of the observation. The increased concentration of EFX and CFX in droppings can be attributed to metabolization and recirculation to other tissues and compartments of the bird as muscle and feathers (Martin et al., 2007; Sampaio de Assis et al., 2016). Peng et al. (2016), analyzed CFX in layer chicken manure and concluded in their risk assessment study that CFX in this waste will not cause environmental risk after a withdrawal period of 28 d. Nevertheless, in our study we estimate through a linear regression analysis that at 56 d the concentration of both analytes, EFX and CFX will be below the 32 μg kg^{-1} (LOQ in matrix of EFX), considering a confidence of 95%.

On the other hand, SCP showed a decrease during the post-treatment period, these results are consistent with those described by Yévenes et al. (2018).

Due to the decrease in the detected concentrations of SCP, TYL, EFX and CFX, a projection was carried out using linear regression, with 95% confidence and considering the LOQ of the analytical methodology as the cut-off point. It was obtained that at d 50, 132 and 56 post-treatment, the concentrations of SCP, TYL and EFX +CFX would be equal to or less than 22.2, 21.9, and 32 μg kg^{-1}, respectively. It was not possible to project the depletion of FF and OTC residues because they did not show a progressive decrease, but rather decreased and increased throughout the sampling points.

The present research show through a controlled study, that different antimicrobials commonly used in poultry industry were eliminated in high concentrations and for a prolonged period through chicken droppings, treated with therapeutic doses of pharmaceutical formulations. Thus, our results demonstrate that this matrix should be considered as a possible

route of antibiotic transfer to the environment such as water and agricultural soil.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101313.

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