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Pharmacokinetics and antibacterial activity of tiamulin after single and multiple oral administrations in geese

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ABSTRACT

Tiamulin is an antibiotic approved exclusively in veterinary medicine, active against G-positive bacteria as well as Mycoplasma spp. and Leptospirae spp. The study was aimed to establish its pharmacokinetics and to evaluate drug effects on resistance in cloacal flora *in vivo* in geese. Eight healthy geese underwent to a two-phase longitudinal study (60 mg/kg single oral administration *vs* 60 mg/kg/day for 4 days) with a two-week wash-out period. Blood samples and cloacal swabs were collected at pre-assigned times. Minimal inhibitory concentration (MIC) has been evaluated for each isolated bacterial species. The pharmacokinetic parameters that significantly differed between the groups were C_{max} ($p = 0.024$), $AUC_{0\text{-}t}$ ($p = 0.031$), $AUC_{0\text{-}inf}$ ($p = 0.038$), $t1/2_{kel}$ ($p = 0.021$), Cl/F ($p = 0.036$), and Vd/F ($p = 0.012$). Tiamulin exhibited a slow to moderate terminal half-life (3.13 h single; 2.62 h multiple) and a rapid absorption (1 h single; 0.5 h multiple) in geese, with an accumulation ratio of 1.8 after multiple doses. An *in-silico* simulation of multiple dosing did not reflect the results of the *in vivo* multiple dosage study. In both treatments, the MIC values were very high demonstrating a resistance (> 64 μg/ml) against tiamulin that can be present prior the drug administration for some strains, or emerge shortly after the commencing of treatment for some others.

1. Introduction

Tiamulin is a semi-synthetic antibiotic possessing a diterpene pleuromutilin structure (Plumb, 2008). It was first isolated from the fungus *Pleurotus mutilis* in the 1950s (Schlünzen et al., 2004). Tiamulin is active against Gram-positive bacteria, as well as Mycoplasma spp. and Leptospirae spp. (Hunt, 2000; Kowalski et al., 2004). Tiamulin demonstrates a bacteriostatic mechanism of action, by obstructing the 50S ribosomal subunit within bacteria, thereby inhibiting the peptidyl transferase enzyme. It can exert a bactericidal effect in very high concentrations against susceptible microorganisms (Plumb, 2008). Tiamulin is approved only in veterinary medicine according to Food and Drug Administration (FDA) and European Medicine Agency (EMA) reports (EMA, 2010; Plumb, 2008). Its approved applications include treating dysentery, pneumonia, and mycoplasma infections in pigs, chickens, and turkeys. An extension to its use in rabbits has been approved as well, allowing for in-feed administration at a dosage equivalent to 11 mg/kg body weight per day for a duration of 21 days (Anonymous, 2008; EMA, 2010; Tang et al., 2012).

Tiamulin is available as premixes and water-soluble powder for pigs and poultry, and as injectable intramuscular formulation for pigs (Anonymous, 2008; EMA, 2010). Pigs and poultry can be given recommended dosages of 50 mg/kg and 320 mg/kg, respectively, in their feed for a duration of up to 6 weeks. Medicated water is recommended in the range 4–25 mg/kg for up to 1 week for pigs, and 30–60 mg/kg for 3–5 days in poultry. The injection (10–20 mg/kg) is administered intramuscularly daily for up to 5 days in porcine (Anonymous, 2008; EMA, 2010). The pharmacokinetics (PK) of tiamulin have been reported in monogastrics such as pigs (Burch, 2005; Laber and Schütze, 1977; Riond et al., 1993), ruminants such as goats, ewes, cows, calves (Ziv

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et al., 1983) and avians such as chickens, turkeys, and ducks (Elazab et al., 2020; Laber and Schütze, 1977; Vinothini et al., 2019; Xiao et al., 2016; Ziv et al., 1983). The most substantial amount of information regarding the PK of tiamulin pertains to swine, wherein it has been shown to undergo 85 % oral absorption, with the peak plasma concentration occurring between 2 and 4 h following a single oral dose (Plumb, 2008). In these species, tiamulin undergoes extensive metabolism and is primarily excreted through faeces (Plumb, 2008).

In addition to the favourable PK profile in various animal species, there have been reports indicating the effectiveness of tiamulin in treating mycoplasma infections in ducks and geese (Elazab et al., 2020; Stipkovits & Szathmary, 2012). Thus, the aim of the study was to establish the PK features of tiamulin after single and multiple oral administrations in geese. Additionally, the authors aimed to investigate the susceptibility/resistance patterns of cloacal commensal microflora in healthy geese concerning tiamulin. The rationale behind this exploration lies in the critical need to comprehend the antibiotic's impact on the natural microbial community residing in the cloaca of geese. With increasing concerns about antibiotic resistance, understanding how tiamulin affects these commensal microorganisms is paramount, shedding light on potential alterations that may occur during the *in vivo* administration of the antibiotic.

2. Materials and methods

2.1. Reagents

The pure powders of tiamulin and phthalimide, both with a standard purity of 99.0 % and the latter used as the internal standard (IS), were purchased from Sigma–Aldrich (Milan, Italy).

All the solvents (acetonitrile, n-hexane and ethyl acetate) were of HPLC grade and were purchased from VWR International (Milan, Italy). Sodium carbonate (Na_2CO_3) powder was purchased from Sigma-Aldrich. Deionized water was produced using a Milli-Q Millipore Water system (Daemstadt, Germany). The mobile phases were filtered through 0.2 μm cellulose acetate membrane filters using a solvent filtration apparatus (Sartorius Stedim Biotech, Goettingen, Germany).

Charcoal Cefoperazone Deoxycholate Agar (CCDA, Microbiol, Cagliari, Italy), Tryptone Bile X-Gluc agar (TBX, Microbiol, Cagliari, Italy), Slanetz and Bartley Agar (SBA, Microbiol, Cagliari, Italy), Rabbit Plasma Fibrinogen (RPF, Microbiol, Cagliari, Italy) and Shaedler agar (SCH, Becton Dickinson, Milan, Italy) media, were used to permit the isolation of Campylobacter spp., Escherichia coli (E. coli), enterococci, staphylococci and anaerobes, respectively.

2.2. Animal treatment

For this study, eight male geese were randomly selected from a larger flock. The animals were approximately 2 years of age, and the body weight ranged between 4.7 and 7.2 kg. The geese were determined to be clinically healthy based on chemistry and hematological analyses performed prior to the study. The animal experiment was carried out in accordance with European law (2010/63/UE) and approved (nr. 78/2021) by the Institutional Animal Care and Use Committee of the University of Lublin (Poland).

The geese were monitored daily by a designated veterinarian (B L-W) who observed their behaviors and appetite.

To acclimate the geese to the study environment, they were kept in a 60 m^2 enclosure with a 9 m^2 indoor shelter for a week. During this time, they were provided with a drug-free pelleted diet twice daily, along with access to water *ad libitum*. An identity code was applied to the left leg for easier identification. The study was longitudinal and divided in two phases with a wash-out period of two weeks: in the first phase, geese were treated with a single PO administration given by crop-gavage, with a dose of 60 mg/kg of body weight (Biomutin 20 % injectable, 200 mg/ml, Monterovet, Poland) (EMA, 1999). In the second phase of the study, the same group was subsequently treated with multiple doses, given by crop-gavage, with a dose of 60 mg/kg/day for 4 days. The injectable formulation was chosen for oral administrations due to its ease of dosing.

Blood samples (about 1.5 ml) were collected from the left-ulnar vein by direct venipuncture. Blood was collected in heparinized tubes, centrifuged at 1500 x *g*, and the resulting plasma was collected and stored at −20 °C. Plasma analysis was performed within 30 days of collection.

Blood was collected at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24 and 48 h in the first phase after the single tiamulin dose.

During the second experimental phase the blood was withdrawn at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24 and 48 h after the last administration of the multiple dose treatment. In addition, blood was withdrawn after the first (24 h), the second (48 h) and the third (72 h) day of treatment, immediately before the administration of the daily dose.

Cloacal swabs (ESwab™, Copan, Italy) were collected in all treatment groups and from all animals prior to tiamulin administration (T0) and after the last dose at, 6, 10, 24, 48, and 72 h. All swabs were placed in sterile tubes and frozen at −20 °C until further processing.

2.3. Plasma sample preparation for tiamulin quantification

With certain adjustments, the analytical approach described by Elazab et al. (2020) was carried out. An aliquot of plasma (0.5 ml) was spiked with 50 μl of IS in a clean 15 ml tube. The samples were vortexed, and 2.5 ml of di Na_2CO_3 and 2.5 ml of a mixture of n-hexane/ethyl acetate (1/3 v/v) were added. Each sample was vortexed, shaken at 60 oscillations/min for 10 min and centrifuged at 4000 x *g* for 10 min. Two ml of the supernatant was collected in a clean tube and dried under nitrogen flow at 40 °C. The residue was reconstituted with 100 μl of tartaric acid 0.1 %. Fifty μl of this latter solution was injected onto the HPLC system.

2.4. HPLC instrumentation

The HPLC system was a LC Jasco consisting of a ternary gradient system (PU 2089), in line degasser (DG-2080-53), autosampler (AS-2055) and an UV detector (UV-975). The chromatographic separation assay was performed with a Luna C18 analytical column (150 × 4.6 mm inner diameter, 3 μm particle size, Phenomenex). The mobile phase consisted of an aqueous solution and acetonitrile in a ratio of 69:31 % at a flow rate of 0.7 ml/min. The aqueous solution was a solution of potassium dihydrogen phosphate (0.01 M) with a pH of 2.8. The optimal wavelength for tiamulin quantification was set at 208 nm.

2.5. Validation of the analytical method

The quantitative HPLC method was fully validated for goose plasma in terms of linearity, intra-day and inter-day precision, recovery, limits of detection (LOD) and lower limit of quantification (LLOQ), according to the EMA guidelines (Anonymous, 2012). Tiamulin and IS stock solutions at 1000 μg/ml were diluted in MeOH to 100 μg/ml and stored at −20 °C. Dilutions at 50, 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 μg/ml were used for tiamulin calibration curve in plasma. Linearity was determined by linear regression analysis, using a calibration curve constructed using replicates ($n = 3$) of control goose plasma samples spiked with tiamulin at the concentrations 0.1–50 μg/ml. The intra-day and inter-day precision were calculated after analysis of nine plasma sample spiked with tiamulin at three different concentrations (0.5, 2 and 10 μg/ml). The intra-day and inter-day precision was expressed as the percentage coefficients of variation (CV, %). The extraction recovery was carried out by comparing the response (in area) of high, middle, low tiamulin standards (0.1, 1 and 10 μg/ml) and the IS. The LOD was estimated as the plasma drug concentration that produced a signal to noise ratio of 3, while LLOQ was determined as the lowest plasma concentration that

produced a signal to noise ratio of 5.

2.6. Pharmacokinetic analysis

The pharmacokinetic analysis was performed using PKanalix™ software (R1; 2023). Tiamulin plasma concentrations were analyzed using a non-compartmental approach for both treatments. Maximum plasma concentration (C_{max}) of tiamulin and the time required to reach it (T_{max}) were acquired directly from the data. The elimination half-life ($t1/2_{kel}$) was calculated using linear least squares regression analysis of the concentration-time curve. The area under the concentration time curve ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$) was calculated using the linear-up log-down rule. Pharmacokinetic estimates were calculated if the individual value of AUC rest % was lower than 20 % of $AUC_{0\text{-}t}$ and R^2 (square of coefficient of determination) of the terminal phase regression line was > 0.85. The apparent volume of distribution (Vd/F) and clearance (Cl/F) were calculated. The accumulation index has been calculated as the ratio between $AUC_{0\text{-}t}$ after the last administration in the multiple dose protocol and $AUC_{0\text{-}t}$ after the single dose (Toutain and Bousquet-Mélou, 2004). A value close to 1 indicates that no accumulation occurred.

The decision to conduct a multiple-dose study of tiamulin in geese was based on mimicking the real-life scenarios with antibiotics, to assess how tiamulin pharmacokinetically behaves in a clinical context, to observe the cumulative effects of the drug, to evaluate the potential for resistance development, and eventually to determine the appropriate dosing regimen that ensures optimal therapeutic outcomes. In parallel, an *in silico* modeling of a daily oral dose regimen of 60 mg/kg/day administered for 4 days was computed applying the superposition principle and assuming first-order kinetics (Gabrielsson and Weiner, 2001). This computational approach enables authors to complement the experimental results, particularly for minor species like geese where conducting a multiple dose study is not always feasible, and ultimately facilitates the exploration of potential outcomes under controlled conditions.

2.7. Bacterial isolation

Swabs were seeded and cultured in different media in order to perform bacterial identification. Only to permit the isolation of Campylobacter spp., swabs were streaked on the surface of the medium present in the petri dish. To perform other identifications, swabs were thawed in peptone water, seeded in selective media and incubated at specific temperature conditions. Colonies isolated on each medium were counted. Ten % was identified by Maldi-TOF (Bruker Diagnostics, Milan, Italy) and subsequently stored at −80 °C. For the detection of Avian Pathogenic *E. coli* (APEC), a pool of all colonies isolated on TBX was suspended in 1 ml of deionized water and subjected to Real-Time PCR using the primers described in the Table 1.

2.8. Susceptibility testing

Antibiotic susceptibility of cryopreserved strains was tested using the minimum inhibitory concentrations (MICs) method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2022) (M100 Performance Standards for Antimicrobial Susceptibility Testing, 32nd Edition). MIC values have been evaluated using the semi-automated system Sensititre (Thermo Fisher Scientific, Milan, Italy) and breakpoints have been expressed in μg/ml. The plates contained ampicillin, penicillin, tilmicosin, bacitracin, lincomycin, doxycycline, tiamulin, tylosin and valnemulin. For the present study, only tiamulin has been considered. Positive (wells without antimicrobial drugs) and negative (wells with a total inhibitory substance) controls have been added to the customized plates. Tiamulin was tested using concentrations ranging from 0.125 to 64 μg/ml.

Table 1
Features of genes and oligonucleotides used to confirm the identification of bacteria strains using Maldi – TOF analysis. The related references are provided in the table.

Gene, Oligonucleotide sequences (5′−3?), fragment sizes (bp), annealing temperatures (°C)	References
kps II,GCG CAT TTG CTG ATA CTG TTG//CAT CCA GAC GAT AAG CAT GAG CA,272,63,	Johnson and Stell (2000)
fimH, TGC AGA ACG GAT AAG CCG TGG//GCA GTC ACC TGC CCT CCG TGG,508,63,	Johnson and Stell (2000)
csgA, ACT CTG ACT TGA CTA TTA CC//AGA TGC AGT CTG GTC AAC,200,50,	Maurer, Brown, Steffens and Thayer (1998)
tsh, GGT GGT GCA CTG GAG TGG//AGT CCA GCG TGA TAG TGG,620,55,	Dozois et al. (2000)
papC, GAC GGC TGT ACT GCA GGG TGT GGC G// ATA TCC TTT CTG CAG GGA TGC AAT A,328,65,	Le Bouguenec, Archambaud and Labigne (1992))
papG, CTG TAA TTA CGG AAG TGA TTT CTG// ACT ATC CGG CTC CGG ATA AAC CAT,	Johnson and Stell (2000)
felA, GGC AGT GGT GTC TTT TGG TG//GGC CCA GTA AAA GAT AAT TGA ACC,270,63,	Johnson et al. (2000)
sfaS, GTG GAT ACG ACG ATT ACT GTG//CCG CCA GCA TTC CCT GTA TTC,240,63,	Johnson and Stell (2000)
sfaDE, CTC CGG AGA ACT GGG TGC ATC TTA C// CGG AGG AGT AAT TAC AAA CCT GGC A,410,55,	Le Bouguenec et al. (1992)
facA, ATG AAG TTA AAA TTC ATC TCC//CTG GTA CTG AAC TTT AAA GG,600,50,	Dziva and Stevens (2008)
afaI, GCT GGG CAG CAA ACT GAT AAC TCT C// CAT CAA GCT GTT TGT TCG TCC GCC G,750,60,	Le Bouguenec et al. (1992)
iutA,GGC TGG ACA TGG GAA CTG G//CGT CGG GAA CGG GTA GAA TCG,300,63,	Johnson and Stell (2000)
cvaC,CAC ACA CAA ACG GGA GCT GTT//CTT CCC GCA GCA TAG TTC CAT,680,63,	Johnson and Stell (2000)
iss,GTG GCG AAA ACT AGT AAA ACA GC//CGC CTC GGG GTG GAT AA,760,61,	Foley, Horne, Giddings, Robinson and Nolan (2000)
cnf1,AGG AAG TTA TAT TTC CGT AGG//GTA TTT GCC TGA ACC GTA A,498,60,	Le Bouguenec et al. (1992)
cnf2,AAT CTA ATT AAA GAG AAC//CAT GCT TTG TAT ATC TA,543,40,	Blanco, Blanco, Alonso and Blanco (1996)

2.9. Statistical analysis

The normality of the data was assessed using a Shapiro–Wilk normality test. The pharmacokinetic parameters were reported as geometric mean and range, except $t1/2_{kel}$ and T_{max}, which were expressed as harmonic mean and median, respectively (Julious and Debarnot, 2000). The paired Student *t*-test was used for the statistical comparison of pharmacokinetic data between the two groups of treatment. Data were analysed with GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Validation of the analytical method

The analytical method demonstrated an optimal specificity and linearity in the concentration range, with R^2 of 0.9994 ($y = 0.0406x + 0.0074$). The method showed linearity in the range 0.5–50 μg/ml. The LLOQ and the LOD were 0.5 and 0.1 μg/ml respectively. The recovery was 90 % (SD 3.4 %). The inter- and intra-day precision were (CV %) $<$ 7.93 %.

3.2. Animals

No adverse reactions were observed in any of the geese during or after drug administration up to 7 days. No deaths have been recorded during and after the experimental sessions.

3.3. Pharmacokinetics

Plasma concentrations of tiamulin were still quantifiable and well above the LLOQ at 10 h after a single dose treatment as well as during the initial day of the multiple treatments (Fig. 1).

The levels of tiamulin in plasma were consistently higher following multiple administrations compared to single doses at most of the time-points, except for the 10 h time point. In both treatments, the tiamulin plasma concentration reached its peak within less than 1 h. The average plasma values collected in the multiple treatment before the next dose were below the LLOQ (24 h), 1.35 (± 0.67) μg/ml (48 h), 2.2 (± 1.15) μg/ml (72 h), and below the LLOQ at 96 h (not reported in Fig. 1). The pharmacokinetic parameters that significantly differed between the groups were C_{max} ($p = 0.024$), $AUC_{0\text{-}t}$ ($p = 0.031$), $AUC_{0\text{-}inf}$ ($p = 0.038$), $t1/2_{kel}$ ($p = 0.021$), Cl/F ($p = 0.036$), and Vd/F ($p = 0.012$). The accumulation ratio was 1.8.

The complete pharmacokinetic parameters of single and multiple treatments are listed in Table 2.

The *in-silico* simulation of multiple dosing did not reflect the experimental situation. Indeed, the plasma concentrations quantified in the *in vivo* multiple dosage study were statistically higher than those computed after the *in silico* analysis (Fig. 2).

3.4. Bacterial isolation

The bacterial strains isolated and identified during the study are individually listed for each treatment group in the supplementary file, providing a comprehensive overview of the study's findings. All isolates have been identified as commensal bacteria. There were no strains of Salmonella spp., Campylobacter spp., or APEC identified in any of the treatment groups or at any time points.

3.5. MICs

Minimal inhibitory concentrations were determined for all isolates at every time point when cloacal swabs were taken. However, only bacteria that were consistently detectable throughout the entire experimental period were taken into account. The results are expressed in Tables 3 and 4. Strains reporting a MIC value higher than 64 μg/ml were evaluated as resistant, considering a range of sensitivity from 0.125 to 64 μg/ml.

In the group treated with a single oral administration of tiamulin, *Aerococcus viridans, Staphylococcus warneri, Staphylococcus sciuri, Staphylococcus borealis* and *Staphylococcus haemolyticus* have been considered.

In the treatment group receiving multiple oral administration of tiamulin, *Clostridium perfrigens, Enterococcus faecalis, Staphylococcus sciuri, Staphylococcus borealis, Staphylococcus equorum* have been considered.

Based on MIC values reported in Tables 3 and 4, some strains had an already very high MIC before the commencement of the treatment at T (0). An increase in MIC values over time post-tiamulin administration was observed only for some strains, and mainly occurred in the single treatment group. Moreover, if MIC values for strains of *Staphylococcus sciuri* and *Staphylococcus borealis* are compared between single and multiple treatment groups, the level of resistance do not seem to increase after multiple dosing.

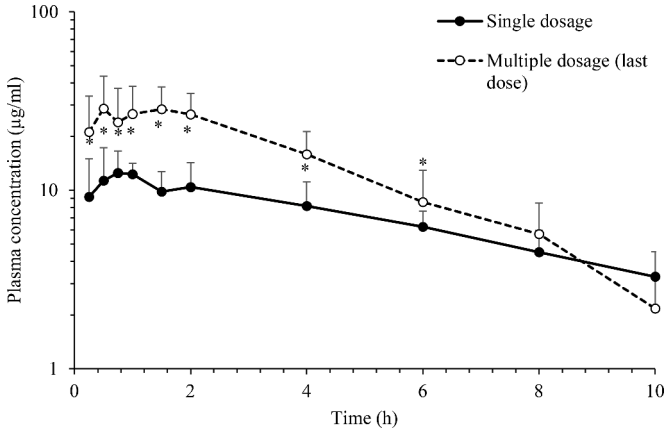

Fig. 1. Semi logarithmic average plasma concentration *vs* time curve of tiamulin after single oral dose (▬●▬, 60 mg/kg) and the multiple oral dose (–○–, 60 mg/kg/day for 4 days) administration in geese ($n = 8$). *; Significant difference in the average plasma concentrations (± SD) for a given time point between the single and multiple administrations.

4. Discussion

One of the aims of this study was the evaluation of the pharmacokinetics of tiamulin after single and multiple oral administrations. The study revealed that orally administered tiamulin to geese was absorbed rapidly with a T_{max} between 0.5 and 1 h. The study reported several differences in PK parameters' values between the treatments. Although an accumulation ratio of 1.8 was found, the AUC after multiple dosing almost doubled. This could be attributed to the decreased Cl/F value (assuming that the F % is the same in the groups). This decline might be a result of drug inhibition involving cytochrome P450 (Fu et al., 2020) or external factors that affected the animals during the second stage. Unfortunately, the lack of a cross-over design does not allow additional speculations. For the multiple administration, the plasma concentrations differed between the *in silico* simulation and the *in vivo* results. These latter values were much higher than the modeled ones, confirming a reduction in clearance. Albeit only one time point of plasma concentration has been evaluated for each day before the last dose, it was reported to increase with time. Consequently, the *in silico* simulation does not seem to fit this drug in geese. More importantly, further studies are warranted to understand the reasons for the increase in tiamulin plasma concentrations when administered in multiple doses. Comparing the present PK parameters with those reported in other species, the AUC values were significantly higher than the values reported for ducks treated with tiamulin directly into the crop at a single dose of 30 and 60 mg/kg (AUC 5.55 mg*h/l and 22.83 mg*h/l, respectively) (Elazab et al., 2020) and broiler chicken receiving tiamulin orally at 40 mg/kg (2.36 mg*h/L) (Vinothini et al., 2019). The values of C_{max} obtained in our study for single and multiple treatment were higher than in ducks, where C_{max} were 0.77 μg/ml for 30 mg/kg and 2.32 μg/ml for 60 mg/kg; and also, higher than in chicken with a C_{max} of 0.73 μg/ml at a dose of 40 mg/kg. Several factors, including interspecies variations, differences in oral administration methods, age, weight, and diet during administration, could have influenced the absorption process among the avian species mentioned above. One of the most likely causes of this difference in the absorption may be related to the different pharmaceutical formulations. Elazab et al. (2020) used an oral solution of tiamulin hydrogen fumarate (Denagard 12.5 %, Elanco Co., Basel, Switzerland) in ducks. While Vinothini used an oral formulation of tiamulin hydrogen fumarate (Vetmulin 80 %, Huvepharma Private Limited, Pune, India) in broiler chicken. Nonetheless, the pharmaceutical preparation employed in this study was an injectable solution administered orally. Theoretically, it exhibits a higher hydrophilicity, potentially leading to an increased and faster absorption.

Tiamulin in the present study showed a relatively short half-life (single dose, 3.13 h; multiple doses, 2.62 h). In ducks and chickens, the respective half-life values were 6.34 h and 4.23 h following oral administration (Elazab et al., 2020; Vinothini et al., 2019). The variation in these values is likely a result of inter-species differences. However, without intravenous studies, it is not possible to conclusively determine whether these differences primarily stem from changes in volume of distribution or clearance.

The results obtained by the susceptibility testing highlighted that MIC values are extremely high in bacteria strains that have been isolated

Table 2
Pharmacokinetic parameters of tiamulin after single (60 mg/kg) and multiple oral dose (60 mg/kg day for 4 days) in geese ($n = 8$).

		Single dose			Multiple dose		
		Geometric mean	Min	Max	Geometric mean	Min	Max
$AUC_{(0\text{-}t)}$	mg h/l	78.26*	49.86	128.28	128.90	84.19	177.97
$AUC_{(0\text{-}inf)}$	mg h/l	89.21*	52.38	141.44	143.38	87.61	206.09
Kel	1/h	0.22	0.17	0.26	0.26	0.21	0.37
$t1/2_{kel}$ ç	h	3.13*	2.69	3.97	2.62	1.88	3.38
Cmax	μg/ml	18.29*	12.35	39.48	38.49	30.54	48.38
Tmax $	h	1	0.25	2.00	0.5	0.25	1.00
Cl/F	mL/g h	0.77*	0.47	1.20	0.47	0.34	0.71
Vd/F	mL/g	3.46*	1.93	4.67	1.76	1.14	2.28

$AUC_{0\text{-}t}$, area under the curve from zero to the last detectable time point; $AUC_{0\text{-}inf}$, area under the curve from zero to infinity; Kel, elimination rate constant; $t1/2_{kel}$, terminal half-life; C_{max}, maximum concentration; T_{max}, time at maximum plasma concentration; Cl/F, apparent plasma clearance; Vd/F, apparent volume of distribution.

* significant difference between the treatments ($P > 0.05$).

$ Median.

ç Harmonic mean.

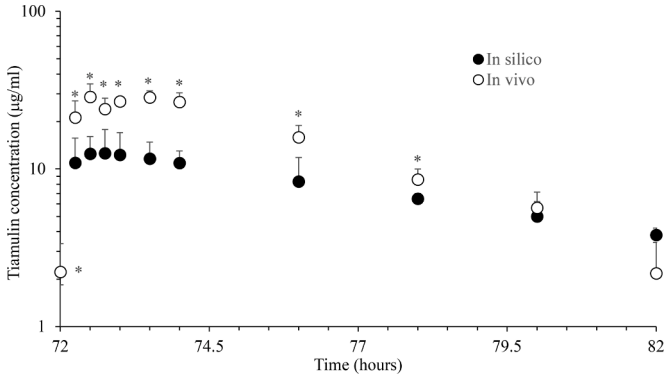

Fig. 2. Semi logarithmic average plasma concentration points *vs* time of tiamulin after the last administration in the *in vivo* and *in silico* multiple administration study ($n = 8$). *; Significant difference in the average plasma concentrations (± SD) for a given time point between the *in vivo* and *in silico* modes.

Table 3
Bacteria strains isolated from cloacal swabs after a single oral administration of tiamulin at 60 mg/kg. Mean minimal inhibitory concentrations (MICs) over time (prior and after the administration of tiamulin) are also expressed.

	MIC (μg/ml)					
Isolates	T 0 h	T 6 h	T 10 h	T 24 h	T 48 h	T 72 h
Aerococcus viridans	0.25	0.25	1	2	2	2
Staphylococcus warneri	4	8	16	16	16	16
Staphylococcus sciuri	>64	>64	>64	>64	>64	>64
Staphylococcus borealis	>64	>64	>64	>64	>64	>64
Staphylococcus haemoliticus	>64	>64	>64	>64	>64	>64

Table 4
Bacteria strains isolated from cloacal swabs after a multiple oral administration of tiamulin at 60 mg/kg. Mean minimal inhibitory concentrations (MICs) over time (prior and after the administration of tiamulin) are also expressed.

	MIC (μg/ml)					
Isolates	T 0 h	T 6 h	T 10 h	T 24 h	T 48 h	T 72 h
Clostridium perfrigens	16	>64	>64	>64	>64	>64
Enterococcus faecalis	>64	>64	>64	>64	>64	>64
Staphylococcus sciuri	>64	>64	>64	>64	>64	>64
Staphylococcus borealis	32	32	32	32	32	64
Staphylococcus equorum	>64	>64	64	64	64	16

in the present study. All strains were identified as non-pathogenic but commensal and, according to authors' knowledge, no previous papers reported the isolation of these bacterial species in geese. The absence of isolation of pathogenic bacteria might be explained by the fact that due to the living and experimental conditions, geese were not in contact with other animals or contaminated environments. Furthermore, an extreme variability has been described all over the world, especially regarding Campylobacter spp., which prevalence can significantly vary accordingly to the geo-localization (Kaakoush et al., 2015).

It has been previously reported that tiamulin and tylvalosin are the most potent and effective antibiotics that can be used to treat infections in geese and waterfowl, both in Europe and in China (Gyuranecz et al., 2020). This is primarily associated with mycoplasmosis, an infection that gives rise to health concerns and economic losses in domestic geese bred for various purposes, such as meat, eggs, feathers, and foie gras production (Stipkovits & Szathmary, 2012). Despite this, data present in literature and derived from specific in-field studies demonstrated an increase of MIC values in Mycoplasma species against tiamulin (Gyuranecz et al., 2020). This trend can be affected and be worsened considering that transmission of resistance can occur. In our study the presence of commensal bacteria has been shown and the vast majority of them demonstrated very high MIC values related to tiamulin. It was previously explained and demonstrated that enterococci can be frequently isolated in poultry and can easily demonstrate multidrug resistant patterns, including against tiamulin (Soodmand et al., 2018). Even if only commensal bacteria have been isolated, this can hide the risk to share resistant genes with pathogenic bacteria or the same commensal bacteria can cause opportunistic infections (Nagamori et al., 2022). Enterococci and Gram positive facultative anaerobic bacteria can be shared between birds and humans (with a high risk of exchange of resistant genes) due to the close contact between the two species and these bacteria can persist for long periods in environment (Asadian et al., 2016; Marinho et al., 2013). All these factors, underline the importance to carefully evaluate a targeted antimicrobial therapy only after the susceptibility testing has been performed, and that also clinically healthy subjects can represent a reservoir for resistant bacteria (Gyuranecz et al., 2020; Soodmand et al., 2018).

This study had a few limitations, including the absence of an assessment regarding the actual transferability of antibiotic resistance genes in the isolated resistant strains of commensal microflora. Additionally, the study did not measure the concentrations of tiamulin in the intestinal environment of geese during the PK evaluation or at the time of cloacal swab collection. Understanding these local concentrations could offer valuable insights into the potential effects on both commensal and pathogenic bacteria. Another possible limitation is the potential influence of storage conditions on the bacteriological isolation outcome of the cloacal swabs.

5. Conclusion

In this study, tiamulin exhibited a slow to moderate terminal half-life and a rapid absorption in geese following oral administration, with an accumulation ratio of 1.8 after multiple doses. The pharmacokinetic profile observed in geese differed from that of other bird species, highlighting inter-species variations. Notably, the pharmacokinetics of tiamulin in multiple doses did not align with the predictions made through *in silico* simulations. The cloacal isolation allowed the identification of various bacterial strains, all commensal. In both treatments, The MIC values were very high, demonstrating a resistance against tiamulin that can be present before the initial administration for some strains, or emerge shortly after the commencement of treatment for some others. These commensal bacterial strains may present minimal health risks to hosts due to their non-pathogenic nature. However, their reduced antibiotic sensitivity raises concern about potential implications for treatment effectiveness, especially if they transform into opportunistic pathogens or transmit their resistance to other organisms. To address these challenges, further research could explore tiamulin efficacy against Mycoplasma species and other key pathogens *in vivo* (diseased state), optimizing accordingly tiamulin's dosing regimen, and potentially considering synergistic treatments to counter the impact of elevated MIC values.

Ethical statement

The animal experiment was carried out in accordance with European law (2010/63/UE) and approved (nr. 78/2021) by the Institutional Animal Care and Use Committee of the University of Lublin (Poland).

Declaration of Competing Interest

The authors declare that there are no conflicts of interest in publishing this work.

Data availability

The data sets used and/or analyzed during the current study are available from the corresponding author, upon request.

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