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RĪGA STRADIŅŠ UNIVERSITY

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Fluoroquinolone Antimicrobial Agent Levofloxacin in Veterinary Medicine and its Pharmacokinetic-Pharmacodynamic Studies

Doctoral Thesis – set of publications – for obtaining the scientific degree "Doctor of Science (*PhD*)"

Sector Group – Medical and Health Sciences
Sector – Basic Medicine
Sub-Sector – Pharmacology

Riga, 2024

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Abstract

In medicine, antibacterial agents represent one of the most important classes of drugs. Fluoroquinolones are among the most widely used antimicrobials. Levofloxacin is a third-generation fluoroquinolone for the use in human medicine, but it has certain applications in veterinary medicine worldwide.

This Thesis is a compilation of four scientific publications aimed at investigating the suitability of levofloxacin as an antimicrobial agent in the veterinary field, and to evaluate its properties and activity in animal species, where it has not been previously comprehensively studied. Narratively, the first publication was a scientific literature review assessing the role of levofloxacin in veterinary medicine, covering the status of levofloxacin use in veterinary medicine worldwide, its antimicrobial activity, resistance problems, pharmacokinetics, tissue residues, adverse effects and drug interactions. The second publication was an experimental evaluation of the levofloxacin pharmacokinetic profiles in 6 clinically healthy rabbits after the 5 mg/kg intravenous, intramuscular and subcutaneous administration using the crossover study design. Additionally, the effects of single levofloxacin administration on tear production and osmolarity were measured. In this study levofloxacin showed high clearance (0.60 mL/g/h) and complete bioavailability after extravascular administration. The third publication assessed the pharmacokinetic profiles of levofloxacin in healthy geese (2 groups of 8 animals, which received 2 mg/kg intravenously and 5 mg/kg orally, respectively) and its depletion profiles in goose muscle, heart, liver, kidney and lung after a single oral dose of 5 mg/kg. In this study levofloxacin clearance was also high (0.28 mL/g/h) and oral bioavailability was also complete. The highest levofloxacin concentrations were found in the liver and kidneys.

The fourth publication was aimed to assess the activity of levofloxacin against the two of the most common bacterial species associated with infections in rabbits. Minimum inhibitory and minimum bactericidal concentrations were determined for 10 isolates of *Pasteurella multocida* and 5 isolates of *Escherichia coli*. A time-killing curve study was performed *in vitro* and *ex vivo* in order to calculate proposed levofloxacin daily doses against *P. multocida* (MIC = 0.015 μg/mL) and *E. coli* (MIC = 0.03 μg/mL) isolates. Doses were calculated as ≤ 0.91 and ≤ 1.43 mg/kg, respectively.

Levofloxacin was well tolerated in most of the study animals, had favourable pharmacokinetic profiles for extravascular administration and was active against bacteria isolated from animals. Despite being used in veterinary medicine in countries outside the EU, as it is categorised by the WHO as the highest priority critically important antimicrobial it is not registered for veterinary use and is not currently allowed to be used in veterinary medicine in the EU. The selection of levofloxacin as a research topic was based on its global ubiquity,

distinct usage status, and broad antimicrobial activity. This makes it a compelling subject for scientific investigation.

Keywords: levofloxacin, pharmacokinetics, pharmacodynamics, rabbit, goose, *Pasteurella multocida*, *Escherichia coli*

Anotācija

Fluorhinolonu grupas pretmikrobu līdzeklis levofloksacīns veterinārmedicīnā un tā farmakokinētikas un farmakodinamikas pētījumi

Antibakteriālie līdzekļi ir viena no svarīgākajām zāļu grupām medicīnā. Fluorohinoloni ir vieni no visplašāk izmantotajiem antibakteriāliem līdzekļiem. Levofloksacīns ir trešās paaudzes fluoorhinolons, kas paredzēts lietošanai humānajā medicīnā, bet tam ir zināms pielietojums arī veterinārmedicīnā.

Šis promocijas darbs ir četru zinātnisku publikāciju kopa, kuru mērķis ir pārbaudīt levofloksacīna piemērotoību izmantošanai veterinārmedicīnā un izvērtēt tā aktivitāti un īpašības dzīvnieku sugās, kurās tas ir mazāk pētīts. Naratīvi, pirmā publikācija ir zinātniskās literatūras apskats, kurā tika novērtēta levofloksacīna loma veterinārmedicīnā, aptverot levofloksacīna lietošanas pašreizējo statusu veterinārmedicīnā pasaulē, tā pretmikrobu aktivitāti, rezistences problēmas, farmakokinētiku, zāļu atliekvielas audos, blakusefektus un zāļu mijiedarbību. Otrā publikācija ir levofloksacīna farmakokinētisko profilu eksperimentāls novērtējums sešiem klīniski veseliem mājas trušiem pēc 5 mg/kg intravenozas, intramuskulāras un subkutānas ievades, izmantojot krustenisko pētījuma dizainu. Turklāt tika mērīta vienreizējas levofloksacīna ievades ietekme uz asaru produkciju un osmolaritāti. Šajā pētījumā pēc ekstravaskulāras ievadīšanas levofloksacīnam bija augsts klīrenss (0.60 mL/g/h) un absolūta biopieejamība. Trešajā publikācijā tika novērtēti levofloksacīna farmakokinētiskie profili klīniski veselām zosīm (divas grupas pa astoņiem dzīvniekiem, kas saņēma attiecīgi 2 mg/kg intravenozi un 5 mg/kg perorāli) un tā izsīkuma profili zosu muskuļos, sirdīs, aknās, nierēs un plaušās pēc vienreizējas perorālas 5 mg/kg devas lietošanas. Šajā pētījumā levofloksacīna klīrenss arī bija augsts (0.28 mL/g/h), un arī biopieejamība bija absolūta. Levofloksacīna koncentrācijas aknās un nierēs bija visaugstākās no analizētiem audiem.

Ceturtās publikācijas mērķis bija novērtēt levofloksacīna aktivitāti pret divām baktēriju sugām, kas visbiežāk ir saistītas ar trušu infekcijām. Minimālā inhibējošā un minimālā baktericīda koncentrācijas tika noteiktas 10 *Pasteurella multocida* un 5 *Escherichia coli* baktēriju izolātiem. Veikts pētījums kura rezultātā iegūtas mikroorganismu nonāvēšanas līknes laikā *in vitro* un *ex vivo*, lai aprēķinātu ieteicamās levofloksacīna dienas devas pret *P. multocida* (MIC = 0.015 μg/mL) un *E. coli* (MIC = 0.03 μg/mL) izolātiem. Devas tika aprēķinātas attiecīgi. ≤ 0.91 un ≤ 1.43 mg/kg.

Levofloksacīns bija labi panesams lielākajā daļā pētījumu dzīvniekiem tam bija labvēlīgs farmakokinētiskais profils ekstravaskulārai ievadīšanai, un tas bija aktīvs pret baktērijām, kas izolētas no dzīvniekiem. Neskatoties uz to, ka tas tiek izmantots veterinārmedicīnā valstīs ārpus ES, PVO to ir klasificējusi levofloksacīnu kā augstākās

prioritātes kritiski svarīgo pretmikrobu līdzekli, kurš nav reģistrēts lietošanai dzīvniekiem un to pašlaik nav atļauts izmantot veterinārmedicīnā ES. Levofloksacīna kā pētījuma tēmas izvēle balstījās uz tā globālo izplatību, atšķirīgo lietošanas statusu un plašo pretmikrobu aktivitāti. Tādējādi tas ir kļuvis par saistošu priekšmetu zinātniskai izpētei.

Atslēgvārdi: levofloksacīns, farmakokinētika, farmakodinamika, trusis, zoss, *Pasteurella multocida, Escherichia coli*

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Abbreviations used in the Thesis

AUC	Area under the concentration vs. time curve
AUMC	Area under the first moment curve
BW	Body weight
CFU	Colony-forming units
C_{max}	Maximal plasma concentration
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of variation
DES	Dry eye syndrome
DNA	Deoxyribonucleic acid
E	Antibacterial effect of levofloxacin
E_0	$\log_{10}$ difference in the bacterial count from 0 to 24 hours of incubation in the control sample
EMA	European Medicines Agency
EU	European Union
F	Bioavailability
FDA	U.S. Food and Drug Administration
HM	Harmonic mean
HPLC	High Performance Liquid Chromatography
I_{max}	Difference between $\log_{10}$ difference in bacterial count between 0 and 24 hours in the control sample ($\log E_0$) and the log10 difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached
IC_{50}	AUC_{24}/MIC producing 50 % of the maximal antibacterial effect
IM	Intramuscular
IS	Internal standard
IV	Intravenous
LOD	Limit of detection
LOQ	Limit of quantification
MAT	Mean absorption time
MBC	Mean bactericidal concentration
MHB	Mueller Hinton Broth
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
MRL	Maximum residue limits
MRT	Mean residence time

N/A	Not applicable
PAE	Post-antibiotic effect
PK	Pharmacokinetics
PO	*per os*
QRDR	Quinolone resistance determining regions
SC	Subcutaneous
SD	Standard deviation
STT	Schirmer Tear Test
t	Time
$t_{1/2}$	Biological half-life
t_{max}	Time to reach maximum drug concentration
TSA	Trypticase soy agar
UV	Ultraviolet
Vd	Volume of distribution
WHO	World Health Organization
λ_z	Slope of the elimination part of the curve
τ	Dosing interval

Introduction

Infectious diseases are a major problem in veterinary medicine and are associated with the need to administer antimicrobial agents to animals by their owners or people in charge. To make antimicrobial therapy more effective, an appropriate dosing regimen, based on pharmacokinetic and pharmacodynamic data is necessary for both infection treatment and limitation of proliferation of resistant bacterial strains (Toutain et al., 2002). This integrative approach is a proven tool for dose optimisation (Toutain & Lees, 2004). It utilises pharmacokinetic parameters such as area under the concentration vs time curve (AUC), maximal plasma concentration (C_{max}) and pharmacodynamic parameters – minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The approach that is based on bacterial time-killing curves, actually, shows more rationality compared with the approach based only on MIC value, which is a static parameter (Ambrose et al., 2007). Fluoroquinolones are frequently used for the treatment of bacterial infections in both human and veterinary medicine. Pharmacokinetic-pharmacodynamic indices of fluoroquinolones indicate the effectiveness of this class of drugs. Levofloxacin, a potent third-generation antimicrobial fluoroquinolone drug, is used both in human clinical practice and to some extent in veterinary medicine (Sitovs et al., 2021). Its use in veterinary medicine is currently limited: it is completely banned for veterinary use in the EU and is only used off-label in companion animals in the USA. Levofloxacin is active against a wide range of Gram-positive and Gram-negative microorganisms and has improved activity, compared to older fluoroquinolones, against streptococci and anaerobic bacteria. The pharmacokinetic parameters of levofloxacin have already been established in several domesticated mammalian species – pets, non-pets, and birds. Several research papers reporting on levofloxacin in non-human animals have been published in recent years (Kilburn et al., 2023; Madsen et al., 2019; Vercelli et al., 2020; Wang et al., 2021), indicating an increasing interest in levofloxacin as an off-label drug for use in animals. This interest is likely due to the fact that many of the currently licenced veterinary antimicrobials do not meet the needs of veterinarians in the management of antibiotic resistant infections (Papich, 2021), and it implies that levofloxacin has promise in the treatment of infections in animals. At the time of the beginning of the work on this Thesis, the published scientific studies on levofloxacin pharmacokinetics and activity in rabbits were scarce, and completely absent in geese.

Aim of the Thesis

To study the rationale for the use of levofloxacin as an antimicrobial agent in veterinary medicine.

Objectives of the Thesis

The following objectives are set to reach the aim of the doctoral thesis:

1. Summarise and review the existing scientific data from the veterinary field related to levofloxacin;
2. Assess and compare the pharmacokinetic profiles of levofloxacin in healthy domestic rabbits after intravenous, intramuscular and subcutaneous routes of administration;
3. Assess the pharmacokinetics of levofloxacin in geese after either intravenous and oral administration, and to evaluate the depletion profile in goose tissues;
4. Explore and evaluate levofloxacin antibacterial activity against common animal infection causative agents *P. multocida* and *E. coli* isolated from rabbits.

Hypothesis of the Thesis

Levofloxacin has the favourable properties to be used as an antimicrobial agent in veterinary medicine.

Novelty of the Thesis

This research identified, compiled and systemically arranged the scientific data on the studies of levofloxacin in the field of veterinary medicine. This information is now published at the international level for use by veterinary practitioners and scientists in making decisions regarding the levofloxacin use.

This is the first study to report pharmacokinetic parameters for levofloxacin in rabbits after the intramuscular and subcutaneous routes of administration, that could potentially be useful for off-label treatment of pet rabbits by their owners.

This is the first study to report the pharmacokinetic profiles of levofloxacin in geese and its depletion profiles from the selected tissues.

This is the first study to evaluate the levofloxacin antimicrobial activity against *P. multocida* and *E. coli* isolated from rabbits and to propose daily doses for extravascular levofloxacin administration.

1 Literature

1.1 Animal species of veterinary pharmacology interest

Veterinary medicine deals with the challenge to treat different types of animals, that include livestock animals, companion animals, working animals, sports animals and laboratory animals. There are more than 40 livestock species as reported by the World Watch List for Domestic Animal Diversity that are domesticated, but exotic animals, such as reptiles, amphibians, birds are also kept as pets and may therefore require treatment (Scherf, 2000). Some species are classified as major (food-producing and non-food producing) and others as minor (food-producing and non-food producing) species by the regulatory agencies in Europe and the USA. In this diversity of species, it is necessary not only to select a drug, but also to determine a rational dosing regimen for the selected drug, including dose rate, inter-dosing interval, duration of treatment and appropriate routes of administration. These peculiarities are dictated by the anatomical, physiological, biochemical and behavioural features of each species. Additionally, animal species show considerable variability in their pharmacokinetic and pharmacodynamic profiles, and the differences are often unpredictable, thus each drug must be investigated on a species-by-species basis to guarantee its effective and safe use (Toutain et al., 2010).

1.2 Pharmacokinetic differences and drug tissue disposition

Interspecies differences in drug disposition or pharmacokinetics are numerous and reflect species differences in the physiological processes involved in the handling of drugs. Pharmacokinetics is a branch of pharmacology that describes the quantitative changes in the drug concentration in the body over time as a function of the dose administered. In order to evaluate the pharmacokinetic profiles, the concentration-time data from biological samples (most commonly used are plasma or serum) are subjected to mathematical models to quantify the processes involved in absorption, distribution, metabolism and excretion of the drug and its metabolites (Buxton, 2023; Riviere & Papich, 2018).

The most important pharmacokinetic parameter is clearance (Cl), as the only parameter measuring the ability of a body (or an organ) to eliminate a drug. It is defined as the rate of elimination by all routes normalised to the concentration of the drug in a biological fluid in which it can be measured. It is one of the determinants of dosage rate. It determines the dose and the dosing frequency necessary to reach the steady-state concentration (Toutain & Bousquet-Mélou, 2004b). The pharmacokinetic parameter, that relates the amount of the drug in the body to its plasma concentration is the volume of distribution (V_d). Several V_d are used due to the fact that the proportionality ratio between the amount of drug in the body and the

plasma concentration has different values depending to the state of drug disposition (V_c is the initial volume of distribution, V_{ss} is the appropriate volume of distribution when plasma concentrations are measured at steady-state, and V_{area} is the V_d when plasma concentrations is measured in pseudo-equilibrium conditions. V_d shows how broadly the drug is mobilised in the body, and is usually small for drugs that remain in the bloodstream, but is large for drugs distributed and bound to tissues (Toutain & Bousquet-Mélou, 2004d). A hybrid parameter, half-life of elimination ($t_{1/2}$ or $t_{1/2}\lambda_z$) is the most reported PK parameter, is defined as the time required to reduce 50 % plasma concentration during the elimination phase. It is estimated by determining the terminal slope of the time-concentration curve and is dependent on both Cl and V_d (Toutain & Bousquet-Mélou, 2004c). The area under the concentration-time curve is the total area under the curve that describes the measured concentration of drug in the systemic circulation as a function of time (from zero to the last measurement point or extrapolated to infinity) and provides an estimate of drug exposure. Importantly, AUC is also used as a measure of bioavailability. Bioavailability (F), defined as 1 or 100 % for an intravenously administered drug, is defined as the fraction (or percentage) of drug that reaches the site of action or a biological fluid (usually the blood in the systemic circulation). For drugs administered via the extravascular routes (e.g. intramuscular, subcutaneous or oral), the bioavailability should be experimentally determined by comparison the extravascular and intravascular AUC values normalised to the dose administered. In case of extravascular administration, the bioavailability will affect the Cl and V_d values, and they will become apparent clearance (Cl/F) and apparent volume of distribution (V_d/F) (Toutain & Bousquet-Mélou, 2004a). Two other pharmacokinetic parameters frequently reported in biological fluids of interest (usually plasma), include maximum drug concentration (C_{max}) and time to reach maximum drug concentration (t_{max}); these values are derived directly from the concentration-time plot (Buxton, 2023).

Under normal physiological conditions, most of the drugs are metabolised to facilitate elimination. The major organs of elimination are kidneys and liver. Thus, parent drug and metabolites are excreted in urine and, to a lesser extent, in faeces. However, due to variable drug distribution and alternative routes of elimination, drugs and their metabolites could also be found in animal products such as edible tissues (muscle, liver) milk, and eggs. Depletion profiles of drug residues are related to pharmacokinetic profiles and administered doses (Lees & Toutain, 2012). The European Medicines Agency (EMA) publishes maximum residue limits (MRL) of selected marker residue (drug or metabolite, or a sum of metabolites) for veterinary drug that is set by the Committee for Medicinal Products for Veterinary Use (Baynes et al., 2016).

1.3 Antimicrobial drugs

To obtain a comprehensive knowledge regarding the veterinary antibiotic drug, its pharmacokinetic profiles in different species should be studied. In addition, information on their biological, toxic effects and pharmacodynamic data are necessary. Pharmacodynamics studies biochemical, cellular, and physiological effects of drugs, including the molecular mechanisms (Manning & Blumenthal, 2023). It is a study of drug exposure in relation to its biological effect on the host, and in case of an antibiotic, this is the bacteria. For antibiotics, the most important pharmacodynamic parameters are the MIC *in vitro*, the post-antibiotic effect (PAE) and the kinetics of bacterial killing. The MIC is the gold standard in microbiology and it is defined as the lowest concentration of antimicrobial that suppresses visible bacterial growth in a defined incubation period (Andrews, 2001). In order to determine the MIC, incubation of a known amount of bacterial inoculum with a range of doubling antibiotic concentrations is necessary for a specified time. According to the MIC value, bacterial susceptibility breakpoints could be classified as "Susceptible", "Susceptible, increased exposure" or "Resistant" according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and "Susceptible", "Intermediate" or "Resistant", according to the Clinical and Laboratory Standards Institute (CLSI) classification (Gaur et al., 2023). A microorganism is categorised as "Susceptible, standard dosing regimen", when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent. "Susceptible, increased exposure" ("Intermediate" according to CLSI) when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection. A microorganism is categorised as "Resistant" when there is a high likelihood of therapeutic failure even when there is increased exposure (EUCAST, 2019). The mean bactericidal concentration (MBC) is be determined by sub-culturing the bacteria-antibiotic suspension from the MIC test on the antibiotic-free media to find the concentration that is required for complete bacterial killing (Andrews, 2001). Killing-kinetic assays are used in order to determine the degree of bacterial killing, and obtained curves follow microbial killing and growth as a function of both time and antibiotic concentration (Ambrose et al., 2007; Mueller et al., 2004).

1.4 Pharmacokinetic-pharmacodynamic integration

The use of pharmacokinetic-pharmacodynamic integration is a proven tool for dose optimisation in order to achieve the particular plasma concentration profile (Toutain & Lees, 2004). This approach is used to determine an optimal dosage regimen for antibiotics, when the two objectives are important – to optimise the clinical efficacy and to minimise the selection of resistant pathogens (Aliabadi & Lees, 2001; Toutain et al., 2002). According to the antibiotic

activity and surrogate indexes used (markers of what is ultimately expected, that is clinical recovery and bacterial eradication), classically, antibiotics are categorised into one of the categories (Mueller et al., 2004):

- time-dependent, (e.g. beta-lactams) The activity of time-dependent antibiotics is based on the duration of time that free plasma concentrations exceed the MIC (T > MIC), of a bacterial pathogen of interest. The longer the drug levels are above the MIC, the more effective is the antibiotic treatment.
- Concentration-dependent antibiotics effect is associated with the maximum free plasma concentration above the MIC (C_{max}/MIC e.g. aminoglycosides) of a bacterial pathogen of interest. For the treatment to be effective the drug concentrations should be maximised and the suppression of bacteria continues after the drug levels fall below the MIC.

Time-dependent antibiotics with a post-antibiotic effect, have characteristics of both classes. The postantibiotic effect is the time period beginning after organisms are exposed to a drug until the survivors begin to multiply to a significant degree (Ambrose et al., 2007). Both time dependent killing and PAE contribute to the antibacterial action, which is dictated by the concentration of a free drug, the time of the contact with bacteria and the mechanism of action. The pharmacokinetic-pharmacodynamic index used to predict the antibiotic effectiveness is the AUC/MIC ratio, where AUC represents a mathematical sum of antibiotic concentrations, frequently calculated as AUC over 24 hours (AUC_{24} or $AUC_{0\text{-}24}$ or $AUC_{0\text{-}24h}$ or *f*AUC, highlighting the free concentration). AUC/MIC is the index used for predicting the efficacy of fluoroquinolones (Martinez et al., 2006; McKellar et al., 2004; Toutain et al., 2002). AUC/MIC (usually AUC_{24}/MIC) ratio is used to calculate the daily dose for an antibiotic. However, MIC is a static parameter, and has certain limitations. MIC determination involves growth of organisms in broth and can fail to simulate *in vivo* conditions in several respects, for example, the time in which microbial inhibition is achieved, the differences in pH and concentrations of several ions in biological fluids (e.g. plasma), compared with broth (Aliabadi & Lees, 2001). Thus, the approach that is based on *in vivo* or dynamic *in vitro* pharmacokinetic-pharmacodynamic systems such as bacterial time-killing curves shows more rationality compared with the approach based only on minimal inhibitory concentration value (Ambrose et al., 2007; Mueller et al., 2004).

1.5 Fluoroquinolone antimicrobial agents

Fluoroquinolones comprise a large group of synthetic antimicrobial agents. Compared to their predecessors, a class of drugs known as quinolones, the latter exhibit an increased

antibacterial activity against *Enterobacteriaceae* and other Gram-negative bacteria (including *Pseudomonas aeruginosa*). Fluoroquinolones have good activity against most Gram-negative bacteria, including *Escherichia coli, Salmonella* spp., *Proteus* spp. and others. Gram-positive bacteria are variably susceptible. *Staphylococcus* species are usually susceptible. Fluoroquinolones are concentration-dependent antibiotics and AUC/MIC ratios of 125–250 hours has been associated with optimal antibacterial action (McKellar et al., 2004), however also lower values, as low as 30–72 hours are reported (Madsen et al., 2019; Wright et al., 2000). Multiple factors can affect the activity of fluoroquinolones, including metal cations (Ca^{2+}, Mg^{2+}, Fe^{2+}) and low pH at the site of infection. There are multiple mechanisms associated with bacterial resistance to fluoroquinolones. Most commonly resistance develops due to the alteration in drug enzyme targets, i.e. mutations in genes that code enzyme DNA gyrase and genes that code enzyme topoisomerase IV. Other resistance mechanisms include decreased drug permeability, increased in fluoroquinolone efflux associated pumps, and plasmid-mediated resistance (Riviere & Papich, 2018).

Fluoroquinolones are among the most important antimicrobials in veterinary medicine, used practically in all species. Currently labelled for the veterinary use in the EU include: enrofloxacin (metabolised to active metabolite ciprofloxacin), danofloxacin, marbofloxacin, orbifloxacin, and pradofloxacin. Difloxacin and ibafloxacin were formerly used in cats, dogs, cattle and poultry. Fluoroquinolone advantages include rapid bactericidal effect, action against a wide spectrum of clinically important bacteria, potency, good tolerance by animals and suitability for administration via different administration routes (Riviere & Papich, 2018). According to the WHO, fluoroquinolones are categorised as “highest priority critically important antibacterial agents”, and should not be used when an effective lower category antimicrobial agent is available (WHO, 2019; EMA, 2020).

1.6 Levofloxacin

The review of levofloxacin properties and use in veterinary medicine is published in the paper “Levofloxacin in veterinary medicine: a literature review” by Andrejs Sitovs, Irene Sartini, and Mario Giorgi. Research in Veterinary Science, 2021 Jul ; 137:111-126. doi: 10.1016/j.rvsc.2021.04.031. PMID: 33964616. Paragraph 1.6 and the material of Annex 5, Tables A1–A7 reference the aforementioned article.

Levofloxacin is a third-generation fluoroquinolone agent. Compared to previous generations of fluoroquinolones, it possesses expanded activity against Gram-positive bacteria and atypical intracellular pathogens (North et al., 1998). Levofloxacin is the optical S- (-) isomer of ofloxacin. Ofloxacin is a racemic mixture, but most of its antimicrobial activity is

due to the S-isomer, which is 32- to 128-fold more potent than the R-isomer. Levofloxacin was developed to take advantage of this antimicrobial potency, which requires approximately half the usual dose of ofloxacin to achieve similar efficacy, with a reduced toxicity profile. Levofloxacin was patented in 1985 by Daiichi Seiyaku Pharmaceutical Co. Ltd in Japan, but was not introduced to the human pharmaceutical market until 1993, when it was produced as oral tablets under the brand name Cravit®. Also, in 1993, Daiichi Sankyo entered into a licensing agreement with Sanofi-Aventis, and levofloxacin was subsequently marketed and sold under the trade name Tavanic®. Since 2010, generic formulations have also been available. Levofloxacin is currently registered for human use by both the United States Food and Drug Administration (US FDA) and EMA, with a variety of formulations available. Oral tablets, oral, injectable and ophthalmic solutions are approved for use in human medicine in the USA (FDA, 2021). Oral tablets, injectable and ophthalmic solutions are approved in the EU (EMA, 2019). Indications for levofloxacin in human medicine include chronic bronchitis, acute sinusitis, inhalational anthrax (post-exposure), nosocomial and community-acquired pneumonia, prostatitis, pyelonephritis, skin and soft tissue infections and urinary tract infections. Levofloxacin is a drug included in the World Health Organization's List of Essential Medicines (WHO, 2021). Generally, all fluoroquinolones are categorised by WHO (WHO, 2019) as "highest priority critically important antimicrobials". Considering the increasing importance of antimicrobial stewardship principles (Lloyd & Page, 2018), antimicrobials of a lower importance category, active against the isolate of interest, should be used in preference to fluoroquinolones. Wherever possible, fluoroquinolone use in veterinary medicine should be based on antimicrobial susceptibility testing in order to mitigate the risk to public health and prevent the spread of bacterial resistance (EMA, 2020).

1.6.1 Levofloxacin physicochemical properties

Levofloxacin (molecular mass 361.37 g/mol) is pharmaceutically available as a hemihydrate, $C18H20FN3O4 \times 1/2H_2O$, (molecular mass 370.38 g/mol). Levofloxacin expresses slightly acidic (carboxylic acid moiety dissociation constant of 6.24) and strongly lipophilic properties, logP = 2.1 (Nowara et al., 1997). It is soluble in dimethyl sulfoxide, dimethyl formamide, glacial acetic acid and chloroform, slightly soluble in ethanol, sparingly soluble in water, and practically insoluble in ether. At a pH range of 0.6–5.8, levofloxacin water solubility is essentially constant at approximately 100 pg/mL (sparingly soluble). Above pH 5.8, the solubility increases rapidly to a maximum at pH 6.7, approximately 272 pg/mL, pKa value 6.25 (North et al., 1998).

1.6.2 Levofloxacin mechanism of action

Levofloxacin shares the same mechanism of action with other fluoroquinolones. Levofloxacin inhibits bacterial DNA gyrase (an enzyme required for DNA replication, transcription, repair, and recombination) and topoisomerase IV, thereby inhibiting the introduction of single-strand breaks on bacterial chromosomes, and resealing them after supercoiling. This prevents bacterial DNA replication and transcription, leading to a bactericidal effect (Riviere & Papich, 2018). Figure 1.1 represents the mechanism of action of levofloxacin.

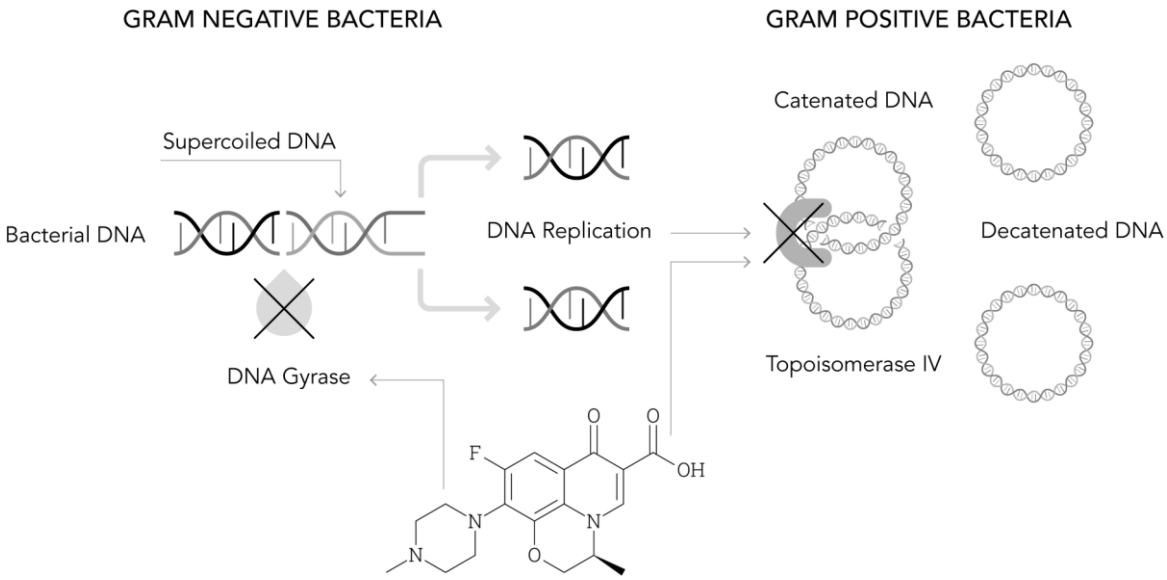

Figure 1.1 **Mechanism of action of levofloxacin against Gram-negative and Gram-positive bacteria**

(Adapted from Herbert et al., 2022)

1.6.3 Use of levofloxacin in human and veterinary medicine

Levofloxacin is effective in the treatment of a variety of infectious diseases. Its spectrum of activity includes Gram-positive aerobic bacteria, Gram-negative aerobic bacteria, some anaerobic bacteria, and other microorganisms including *Chlamydia* spp., *Mycoplasma* spp., and *Mycobacterium* spp. Similar to human formulations of levofloxacin, veterinary formulations are available as oral and parenteral forms in non-EU countries (see Annex 5, Table A1). These products are used for farm animals with infectious diseases (Al Masud et al., 2020). Лексофлон (Lexoflon), for example, is indicated for the treatment of infections caused by the microorganisms listed in Table 1.1.

Table 1.1

Reported antimicrobial spectrum of activity of veterinary levofloxacin formulation Лексофлон (Lexoflon) (NITA-FARM, 2022)

Gram-positive	Gram-negative	Atypical intracellular
Clostridium spp.	*Bacteroides* spp.	*Chlamydia* spp.
Listeria monocytogenes	*Campylobacter* spp.	*Mycoplasma* spp.
Staphylococcus spp.	*Enterobacter* spp.	*Rickettsia* spp.
Streptococcus spp.	*Escherichia coli*	
	Fusobacterium spp.	
	Haemophilus spp.	
	Moraxella spp.	
	Pasteurella spp.	
	Proteus spp.	
	Pseudomonas aeruginosa	
	Salmonella spp.	

To achieve maximum therapeutic efficacy, adequate susceptibility of the microorganism to the therapeutic agent is required. Susceptibility and MIC values for levofloxacin have been reported for multiple microorganisms isolated from animal sources, however as no veterinary specific breakpoint values are available for levofloxacin, human medical breakpoints have been used. It is of great importance that, according to the Clinical and Laboratory Standards Institute VET09 report (CLSI, 2019b), susceptibility test results interpretations based on human breakpoints should be made with low confidence in the correlation between *in vitro* results and clinical outcomes in animals. Authors of most of the publications reported in Tables A2 and A3 of Annex 5 used susceptibility breakpoint values from the Performance Standards for Antimicrobial Susceptibility Testing 28th edition, supplement M100 (CLSI, 2018b). According to these standards, levofloxacin MIC breakpoints for most of the microorganisms are as follows: susceptible = ≤ 2 μg/mL, intermediate = 4 μg/mL, resistant = ≥ 8 μg/mL; and for the disk diffusion method, zone diameter breakpoints: susceptible = zone diameter ≥ 17 mm, intermediate = 14–16 mm, resistant = ≤ 13 mm. CLSI rationale document suggests different

breakpoint values for *Enterobacteriaceae* and *Pseudomonas aureginosa*: *Enterobacteriaceae* susceptible = ≤ 0.5 μg/mL, intermediate = 1 μg/mL, resistant = ≥ 2 μg/mL; *P. aureginosa* susceptible = ≤ 1 μg/mL, intermediate = 2 μg/mL, resistant = ≥ 4 μg/mL (CLSI, 2019a).

1.6.4 Levofloxacin antimicrobial resistance problem

Microbial resistance to fluoroquinolones may result from mutations in defined regions of DNA gyrase or topoisomerase IV (i.e. quinolone resistance determining regions (QRDRs) – *gyrA* and *parC*) or altered efflux. The development of microbial resistance to levofloxacin has been studied in human medicine, however there is limited research in other animal species. Mutations in microbial genes isolated from animals associated with increased resistance to levofloxacin, such as an increase in efflux pump expression, have been documented in molecular studies in a variety of microorganisms, including *Escherichia coli* (Cheng et al., 2020; Liu et al., 2012), *Riemerella anatipestifer* (Sun et al., 2012), *Salmonella* spp. (Kang & Woo, 2014; Kim et al., 2013), *Haemophilus parasuis* (Zhao et al., 2018), and *Staphylococcus aureus* (Suzuki et al., 2016). A pharmacokinetic-pharmacodynamic study in goats identified an increase in levofloxacin resistance of *E. coli* isolated from goats within 48 hours of low dose (2 mg/kg bodyweight) parenteral levofloxacin administration, however the authors did not investigate the underlying mechanism for this finding (Vercelli et al., 2020).

1.6.5 Antimicrobial activity of levofloxacin against Gram-negative microorganisms

Gram-negative bacterial susceptibility to levofloxacin was reported against *Acinetobacter* spp. (Gurung et al., 2013; Kanaan et al., 2020), *Aeromonas hydrophilia* (Pauzi et al., 2020; Stratev et al., 2013), *Brucella* spp. (Morales-Estrada et al., 2016), *Bordetella* spp. (Beach et al., 2012), *Citrobacter freundii* (Goldberg et al., 2019), *Escherichia coli* (Ajayi et al., 2011; Anes et al., 2020; Bandyopadhyay et al., 2012; Batabyal et al., 2018; Benameur et al., 2019; Bhadaniya et al., 2019; Boyal et al., 2018; Chen et al., 2014; Cheng et al., 2020; Hashem et al., 2022; Hussein et al., 2022; Ibrahim et al., 2019; Inoue et al., 2013; Jassim & Shareef, 2023; Jiang et al., 2011; Karim et al., 2020; Liu et al., 2012; Mahmud et al., 2018; Mohanty et al., 2013; Onanga et al., 2020; Panda et al., 2010; Prajapati et al., 2020; Sitovs et al., 2023; Subedi et al., 2018; Tanzin et al., 2016; Zhao et al., 2005), *Enterobacter* (Goldberg et al., 2019), *Francisella tularensis* (del Blanco et al., 2004), *Fusobacterium* spp. (Bhadaniya et al., 2019), *Haemophilus parasuis* (Zhang et al., 2014; Zhao et al., 2018; Zhu et al., 2018), *Helicobacter suis* (Berlamont et al., 2019), *Klebsiella pneumoniae* (Arya Kumar et al., 2020), *Pasteurella multocida* (Sitovs et al., 2023), *Pseudomonas* spp. (Bai et al., 2019; Bhadaniya et al., 2019; Eraky et al., 2020; Farghaly, 2017; Ledbetter et al., 2007; Park et al., 2020; Qi et al., 2014;

Rubin et al., 2008), *Proteus* spp. (Huang et al., 2013; Marques et al., 2019; Pathirana et al., 2018; Sun et al., 2020), *Shigella sonnei* (Zhu et al., 2018), *Salmonella* spp. (Badr et al., 2020; Elfeil et al., 2020; Huamán et al., 2020; Karim et al., 2020; Rahman et al., 2016; Tamuly et al., 2011), *Vibrio* spp. (Li et al., 2018; Özer et al., 2008) and others (Da Silva et al., 2013). Susceptibility data expressed as reported (i.e. if only the percentage of resistant strains were reported, the percentage of sensitive strains was not calculated herein) is presented in Annex 5, Table A2. Some data is missing or incomplete, because it was not reported in published papers (e.g. MIC value); the same approach has been taken for other tables in the Annex 5. Studies that evaluated the susceptibility of *E. coli* isolated from animals to levofloxacin, sometimes report almost complete resistance (Anes et al., 2020; Benameur et al., 2019), however highly sensitive strains were also reported (Bandyopadhyay et al., 2012; Karim et al., 2020). Additionally, an increase in the percentage of resistant *E. coli* strains from 1993 to 2013 was reported (Chen et al., 2014). Many studies investigating the efficacy of levofloxacin in other Gram-negative infections have been undertaken in mouse models. In these models, levofloxacin results in 100 % animal survival, but fails to fully eradicate *Burkholderia mallei* (MIC 2.5 μg/mL) (Judy et al., 2009). Levofloxacin alone and in combination with rifampicin is effective in *Brucella melitensis* infections (Safi et al., 2013, 2014). Together with anti-TNF-α antibodies levofloxacin is effective against enterohaemorrhagic *E. coli* (Isogai et al., 2001). Also, in the mouse, levofloxacin demonstrates efficacy against a seemingly lethal dose (intra-nasal infection with approximately 99 colony-forming units) of *Francisella tularensis* and subsequent antibody development post-treatment (Klimpel et al., 2008).

1.6.6 Antimicrobial activity of levofloxacin against Gram-positive and other microorganisms

Gram-positive bacterial susceptibility to levofloxacin was reported against *Staphylococcus* spp. (Agnoletti et al., 2014; Bhadaniya et al., 2019; Kang & Woo, 2014; Lozano et al., 2011; Mohanty et al., 2013; Ruscher et al., 2010; Salauddin et al., 2020; Sasaki et al., 2007; Sharma et al., 2020; Tanzin et al., 2016; Upadhyay & Kataria, 2009; Van den Eede et al., 2013; Vanni et al., 2009; Zdolec et al., 2016; Zhou et al., 2017), *Enterococcus* spp. (Davedow et al., 2020; Liu et al., 2023), *Lactobacillus* spp. (Saleem et al., 2018), *Actinomyces bowdenii* (Sherman et al., 2013), *Streptococcus* spp. (Bhadaniya et al., 2019; Eisenberg et al., 2017; Ichikawa et al., 2020; Mohanty et al., 2013; Soares et al., 2014; Yang et al., 2020), *Clostridium difficile* (Alvarez-Perez et al., 2014; Álvarez-Pérez et al., 2013; Álvarez-Pérez et al., 2015; Bandelj et al., 2017; Rodriguez-Palacios et al., 2006; Thitaram et al., 2016), *Bacillus* spp., *Micrococcus* spp., *Corynebacterium* spp. (Bhadaniya et al., 2019), *Mycoplasma bovis* (Mustafa et al., 2013), *Mycobacterium avium* (Kanegi et al., 2019) and others (Bajaj et al., 2018). The

susceptibility of Gram-positive and atypical microorganisms to levofloxacin is presented in Annex 5, Table A3. The majority of investigated microorganisms are reported to have susceptibility to levofloxacin, e.g. a retrospective study of dog osteomyelitis showed that less than 10 % of various isolated microorganisms were resistant to this drug (Siqueira et al., 2014). However, there are some exceptions. Multiple studies (Alvarez-Perez et al., 2014; Álvarez-Pérez et al., 2013; Rodriguez-Palacios et al., 2006) have indicated complete clostridial resistance to levofloxacin. Complete *Staphylococcus pseudintermedius* resistance to levofloxacin in dogs was reported as well (Ruscher et al., 2010; Sasaki et al., 2007). Studies into the susceptibility of *S. aureus* have reported mixed results: on the one hand, oral administration of levofloxacin was more effective than ciprofloxacin in rabbits with S. aureus abscesses (Fernandez et al., 1999), on another hand ophthalmic administration in rabbits was not effective in the reduction of keratitis caused by a resistant *S. aureus* strain (Tungsiripat et al., 2003). Similarly, an *in vitro* pharmacokinetic model of bulbar conjunctiva of rabbits reported a stronger bactericidal effect of 1.5 % levofloxacin ophthalmic solution compared to 0.5 % solution against different MIC *S. aureus* strains (Suzuki et al., 2016). Levofloxacin was identified as the fluoroquinolone of choice in elephant tuberculosis (Backues & Wiedner, 2019), despite the fact that earlier study reported unsuccessful treatment of *Mycobacterium tuberculosis* infection in captive elephants due to poor compliance and adverse effects (Miller et al., 2018). Rabbits infected with *Bacillus anthracis* (MIC 0.12 μg/mL) showed high survival rates, suggesting that intravenous levofloxacin is an effective therapeutic agent against inhalational anthrax (Yee et al., 2010). Oral administration of levofloxacin was effective in the anthrax model in Rhesus monkeys, where an initial dose of 15 mg/kg followed by 4 mg/kg every 12 hours prevented morbidity and mortality and did not cause development of microbial resistance (Kao et al., 2006). Topical levofloxacin formulation containing, miconazole, and dexamethasone was found to be effective in external otitis management in cats (Bastos et al., 2019). In buffalos intrauterine coadministration of levofloxacin with ornidazole and α-tocopherol was effective in treating and preventing postpartum affection (Markandeya et al., 2011).

1.6.7 Adverse effects of levofloxacin

Levofloxacin side effects have been comprehensively documented in human medicine, and encompass common gastrointestinal effects (nausea, diarrhoea, constipation), headache, insomnia, dizziness, and rare, but severe tendinitis and peripheral neuropathy (Liu, 2010). However, reports of side effects in animals are limited. Most of the levofloxacin studies in veterinary medicine performed a single dose administration (dose range 2–810 mg/kg body

weight) and not all of them reported on side effects. Of those that did report on side effects, most suggested a lack of side effects associated with levofloxacin treatment (Aboubakr, 2012; Aboubakr & Soliman, 2014; Aboubakr et al., 2014; Albarellos et al., 2005; Bisht et al., 2018; Casas et al., 2019; Dumka & Srivastava, 2006; Goudah, 2009; Goudah & Abo-El-Sooud, 2009; Goudah & Hasabelnaby, 2010; Landoni & Albarellos, 2019; Lee et al., 2017; Patel et al., 2012; Patel et al., 2012; Sartini et al., 2020; Sartini et al., 2021; Urzua et al., 2020; Varia et al., 2009; Vercelli et al., 2020). Transient vomiting, soft faeces, diffuse erythema, pruritus, and signs of depression were reported in two of the animals in the study following intravenous administration of 15 mg/kg levofloxacin in dogs (Madsen et al., 2019). High single doses (810 mg/kg) of oral levofloxacin have also been reported to cause gastrointestinal side effects in female rats (Watanabe et al., 1992). Interestingly, the same study found that a much lower single oral dose (50 mg/kg) of levofloxacin in rabbits also caused gastrointestinal issues (reduction in food intake and body weight). Similarly, a toxicological study in broiler birds reported that a dose of 60 mg/kg bodyweight (considered therapeutic) was associated with gastrointestinal and haematological adverse effects, while supratherapeutic doses caused more severe gastrointestinal and haematological toxicity as well as muscle weakness and loss of body weight (Kumar et al., 2009). Despite the few reports of overt side effects in animals, molecular studies have found adverse effects of levofloxacin on various tissues, especially with extended dosing regimens. A reduction in antioxidant activity in rabbits was reported following 21 days of oral treatment with 10 mg/kg bodyweight levofloxacin (Khan & Rampal, 2013). In rats, oral administration of levofloxacin for 4 weeks revealed cytotoxic but not genotoxic effects (Al-Soufi & Al-Rekabi, 2018). Oral administration of levofloxacin for 30 days at doses from 9.37 to 37.5 mg/kg body weight resulted in deleterious effects on the liver, kidney and testes in mice (Ara et al., 2020), however, no clinical signs were found of levofloxacin-induced liver toxicity after oral administration of 40 mg/kg bodyweight in rats for just two weeks (although liver enzymes associated with liver damage and oxidative stress markers were elevated) (Farid & Hegazy, 2020). Some experimental reports and case studies have reported other potential effects of levofloxacin in animals. An anxiety-like effect in rats, and a reduction in sleep in mice were observed (Erden et al., 2001), this study also suggested that levofloxacin had analgesic activity in mice. Finally, a case report reported the development of a corneal plaque containing levofloxacin in a dog, following administration of levofloxacin eye drops for a period of 2 weeks (Park et al., 2015).

1.6.8 Recap of levofloxacin pharmacokinetics in veterinary species

Levofloxacin pharmacokinetic profiles have been established for different animal species, however these used different analytical techniques for levofloxacin concentration detection (microbiological assay, HPLC with fluorescence detection, HPLC with UV/Vis detection, HPLC/MS), different experimental protocols and different pharmacokinetic modelling approaches. This makes comparing such data challenging. Some authors indicate that the pharmacokinetics of levofloxacin is best described by a two-compartmental pharmacokinetic model (Czyrski et al., 2015; Goudah & Abo-El-Sooud, 2009; Ram et al., 2008), while others applied a non-compartmental approach (Lee et al., 2017; Sitovs et al., 2020; Vercelli et al., 2020). Pharmacokinetic profiles of levofloxacin have been reported in dogs (Landoni & Albarellos, 2019; Madsen et al., 2019; Urzua et al., 2020; Yin et al., 2011), cats (Albarellos et al., 2005), giant pandas (Wang et al., 2021), rabbits (Czyrski et al., 2015; Destache et al., 2001; Sitovs et al., 2020), guinea pigs (Edelstein et al., 1996), rats (Cheng et al., 2002; Dharuman et al., 2010; Hurtado et al., 2014), mice (Yarsan et al., 2003), cattle (Dumka & Srivastava, 2007; Dumka & Srivastava, 2006; Kumar et al., 2009; Kumar et al., 2012), buffalo (Ram et al., 2008), goats (Goudah & Abo-El-Sooud, 2009; Ram et al., 2011; Vercelli et al., 2020), sheep (Durna Corum et al., 2020; Goudah & Hasabelnaby, 2010; Patel et al., 2012; Sartini et al., 2020), camels (Goudah, 2009), horses (Goudah et al., 2008) and monkeys (Hemeryck et al., 2006; Kao et al., 2006; Nelson et al., 2010). In bird species, levofloxacin pharmacokinetics was assessed in chicken (Bisht et al., 2018; El-Banna et al., 2013; Lee et al., 2017; Patel et al., 2012; Varia et al., 2009), turkeys (Aboubakr et al., 2014), quails (Aboubakr, 2012), geese (Sartini et al., 2021), and ducks (Aboubakr & Soliman, 2014).

Comparison of the main pharmacokinetic parameters in mammalian species is presented in Annex 5, Tables A4 and A5. The fastest clearance in mammals was observed in rabbits (Sitovs et al., 2020) and sheep (Patel et al., 2012), and the longest elimination in cats (Albarellos et al., 2005). The fastest clearance in birds was observed in broiler chickens in one of the studies (El-Banna et al., 2013), however other studies on chickens have shown slower clearance values. Longest elimination was reported in broiler chicken study (Lee et al., 2017). Of other poultry, Bilgorajska geese had the longest elimination time (Sartini et al., 2021).

1.6.8.1 Plasma protein binding

Plasma protein binding of levofloxacin in animals is generally lower than reported value in humans 38 % (Fish & Chow, 1997). The *in vitro* plasma protein binding of levofloxacin has been assessed in various species it is summarised in Annex 5, Table A6. The highest reported plasma protein binding was 45.5 %, in rats (Hurtado et al., 2014), and the lowest 4.2 %, in

broiler chickens (El-Banna et al., 2013). Protein binding was never high enough to significantly affect levofloxacin pharmacokinetics.

1.6.8.2 Tissue disposition and residues

Annex 5, Table A7 presents levofloxacin disposition in poultry tissues, including suggested withdrawal times. Withdrawal times for registered veterinary products containing levofloxacin are reported in Table A1. Multiple pharmacokinetic studies have also reported on the distribution of levofloxacin in the tissues of various mammalian species. In rats, levofloxacin reached its highest concentration (2.31 μg/mL) in prostate dialysate fluid following intravenous administration of 7 mg/kg bodyweight levofloxacin (Hurtado et al., 2014). After a single intravenous administration of 0.5 μmol/kg to rats (0.18 mg/kg), the highest levofloxacin concentration within 3 minutes in the kidney medulla – 10.4 nmol/g (3758 μg/kg), followed by the kidney cortex – 6.2 nmol/g (2241 μg/kg) and the lowest concentration in brain – 0.03 nmol/g (11 μg/kg) (Ito et al., 1999). Investigation of the distribution of levofloxacin in several tissues in sheep (muscle, liver, kidney, heart, lung), following intravenous administration of the drug daily for five days showed the highest reported concentration of levofloxacin was in the kidney, and all tissues had detectable levels of levofloxacin 48 hours after the final dose was administered. This study also reported no accumulation of levofloxacin in the plasma or organs (Sartini et al., 2020). Levofloxacin was found to penetrate better than other fluoroquinolones into the lungs of mice (Klesel et al., 1995) and to accumulate in the lung of guinea pigs (Edelstein et al., 1996). Ocular concentrations reached their highest levels 1 hour post oral administration of 20 mg/kg bodyweight levofloxacin in rabbits. In this study, ocular concentration was higher in pigmented rabbits compared to albino ones (Mochizuki et al., 1994). After ophthalmic administration, comparable concentrations in extraocular tissues, eyelid, conjunctiva and cornea were reported (Sakai et al., 2019). Given the importance of minimising antibiotic residues in milk for human consumption, levofloxacin distribution into and elimination from the milk has been studied. As a weak organic acid, levofloxacin is expected to rapidly diffuse into the milk (Ram et al., 2008). It is therefore unsurprising that studies have investigated this phenomenon in milk-producing animals. Levofloxacin distribution in goat milk was evaluated (Goudah & Abo-El-Sooud, 2009; Ram et al., 2011). After the administration of 4 mg/kg bodyweight, reported milk protein binding was 37 % and a good penetration rate from blood to milk after intravenous and intramuscular administration. AUC_{milk}/AUC_{plasma} ratios are 0.81 and 1.01 respectively. Elimination half-life from milk was similar regardless of administration route, and shorter than 4 hours (Goudah & Abo-El-Sooud, 2009). Longer elimination half-life from milk in mastitic

goats (7.5 hours) versus in healthy goats (4.5 hours) after intravenous administration of 10 mg/kg bodyweight levofloxacin was reported, highlighting the importance of considering potential differences in elimination induced by concurrent disease (Ram et al., 2011).

1.6.8.3 Metabolism

Formation of metabolites is negligible in view of levofloxacin antimicrobial activity in humans, with no active metabolites identified. Very limited data is available regarding the metabolic pathways of levofloxacin in animals. Minimal formation of levofloxacin beta-glucuronide (M1, not identified in humans), desmethyl-levofloxacin (M2), and levofloxacin-N-oxide (M3) reported in rats, dogs and monkeys (Fish & Chow, 1997). Similar results were also reported in Rhesus monkeys, with a further two unnamed metabolites also identified. The authors proposed that metabolites were formed directly from levofloxacin by N-demethylation, N-oxidation and glucuronide conjugation. All metabolites were in far lower concentrations than the parent compound (Hemeryck et al., 2006).

1.6.8.4 Bioavailability

Relative bioavailability of levofloxacin is among the highest of all fluoroquinolones, reported as over 100 % in multiple studies (Lee et al., 2017; Madsen et al., 2019; Sartini et al., 2020), and thus considered complete. Complete oral bioavailability was reported in sheep (Sartini et al., 2020), dogs (Madsen et al., 2019; Yin et al., 2011), and chickens (El-Banna et al., 2013; Lee et al., 2017). The lowest oral bioavailability was reported after administration of a sustained-release formulation in dogs (Yin et al., 2011). Bioavailability following intramuscular and subcutaneous administration is variable between species, with the range of intramuscular bioavailability being 57–106 %, and subcutaneous bioavailability 80–119 %. Average bioavailability value exceeds 90 % in multiple studies. The lowest parenteral bioavailability was reported in cattle calves – 60 % after intramuscular administration. Similarly, the reported range of average oral bioavailability in animals is 42–123 % (See Annex 5, Tables A4 and A5).

1.6.8.5 Excretion

In Rhesus monkeys, levofloxacin is rapidly excreted unchanged, mainly in urine (58-65 %), while minor metabolites (reported above) represented < 5 % in urine (Hemeryck et al., 2006). In the same study, a minor fraction of administered levofloxacin was excreted in faeces (7.4–14.7 %) with approximately 1–2 % being the parent compound and 4–7 % an unknown levofloxacin metabolite. Urinary excretion in cattle and goats has been investigated in several studies. Levofloxacin was detectable in urine 24 hours post intravenous administration in calves

(Dumka & Srivastava, 2007), whereas in goat urine up to 36 hours after intravenous administration (Goudah & Abo-El-Sooud, 2009). Urinary levofloxacin concentrations up to 18 times higher than levels in the plasma and milk (Goudah & Abo-El-Sooud, 2009). Higher urinary excretion of levofloxacin in febrile calves compared to healthy calves (Kumar et al., 2009).

1.6.8.6 Pharmacokinetic interactions of levofloxacin with other compounds

The impact of co-administration of levofloxacin with other drugs or natural products on levofloxacin pharmacokinetics has been reported in several research papers. Sucralfate pre-treatment significantly decreased oral levofloxacin absorption in mixed-breed dogs, reducing maximum plasma concentration from 1.95 μg/mL to 0.57 μg/mL, and bioavailability from 72 % to 32 % (Urzua et al., 2020). Co-administration of levofloxacin with sunitinib in rabbits results in an increase in the levofloxacin elimination rate constant and decreased its half-life (Czyrski et al., 2015). Co-administration of levofloxacin with either tolfenamic acid or flunixin meglumine resulted in slower levofloxacin elimination (Durna Corum et al., 2020). Pretreatment of broiler chickens with amprolium and toltrazuril before levofloxacin administration reduces bioavailability and distribution to the internal organs (El-Banna et al., 2013). A number of medications have been reported to not interfere with levofloxacin pharmacokinetics: cyclosporin pretreatment does not affect levofloxacin biliary distribution in rats (Cheng et al., 2002), administration of intramuscular paracetamol does not affect the pharmacokinetics of levofloxacin in cattle calves (Dumka & Srivastava, 2007) and intramuscular ketoprofen does not influence levofloxacin pharmacokinetics in goats (Jatin et al., 2018). Pretreatment with trikatu (mix of plant extracts *Piper nigrum*, *Piper longum*, and *Zingiber officinale*), however, increases levofloxacin bioavailability in the goat (Patel et al., 2019).

1.7 Rabbits as subjects for levofloxacin study

Rabbits (*Oryctolagus cuniculus*) have a small role as food-producing veterinary species, they are classified as minor food-producing species however, they are frequently kept as companion animals (D'Amico et al., 2022; Toutain et al., 2010). Like other small mammals, rabbits are susceptible to a variety of microbial infections, with the most common infective organisms identified as *Pasteurella* spp., *Enterobacteriaceae* spp., *Streptococcus* spp., and *Staphylococcus* spp. (Percy & Barthold, 2013; Rougier et al., 2006). *Pasteurella multocida* in rabbits can cause productive rhinitis, conjunctivitis, otitis, subcutaneous abscesses, bronchopneumonia, metritis and pyometra (EFSA, 2021; Jekl, 2021; Percy & Barthold, 2013). *Escherichia coli* infection in rabbits is generally associated with neonatal and post-weaning

colibacillosis, accompanied by gastrointestinal tract pathology (ANSES, 2020; El-Ashram et al., 2020).

Previously in rabbits, the pharmacokinetics of levofloxacin have been studied only after intravenous (IV) administration, with limited samples taken following drug administration, the animals in that study were infected with *Streptococcus pneumoniae* for use as a model for meningitis; thus, the kinetics obtained may have been altered due to infective processes (Destache et al., 2001). Regardless, the full pharmacokinetic profile of levofloxacin in healthy rabbits has not been established before the study performed in the scope of this Thesis. IV administration requires specific administration skills and is unlikely to become routinely used in rabbits as prey species are less tolerant of handling than predator species (Giguère et al., 2013). In contrast, intramuscular (IM) and subcutaneous (SC) routes of administration are suitable for use in rabbits (Shellim, 2011) as those methods are easily performed, minimizing handling of and stress to the animal. Thus, IM or SC administration in rabbits is more convenient and faster for veterinary practitioners, and, in exceptional cases, the drug could even be administered by the owner. Despite all 3 routes of administration being parenteral, the pharmacokinetics of each route could differ, affecting the onset and duration of action and bioavailability, thus the IM and SC levofloxacin administration was also performed.

Rabbits have been used as a model to test the effects of eye drops containing fluoroquinolones (Krustev et al., 2014; Sakai et al., 2019). Prior to the study in the scope of this Thesis, there were no data on the effect on tear production and quality after parenteral administration of levofloxacin or any other fluoroquinolone approved for systemic use in rabbits. The ocular surface requires a tear film to cover the eye surface in order to maintain eye health and function. Dry eye syndrome (DES) occurs as a result of decreased tear production or increased tear film evaporation. DES in humans and animals can lead not only to discomfort but also corneal and conjunctival damage. There are reports in humans and animals showing that systemic use of drugs such as beta-blockers, angiotensin-converting enzyme inhibitors, diuretics, and antimicrobials have ocular side effects, and most of those drugs have been reported to cause DES (Blomquist & Palmer, 2011; Rajaei et al., 2015; Shirani et al., 2010). There is evidence that systemic administration of other antimicrobial agents — sulphonamides — can decrease tear production in rabbits (Shirani et al., 2010). In the scope of this Thesis the study on rabbits was used to establish and compare the pharmacokinetic profiles of levofloxacin after single administration via IV, IM, and SC routes in healthy rabbits. Additionally, the effects of levofloxacin administration on tear quantitative and qualitative parameters were assessed.

1.8 Geese as subjects for levofloxacin study

According to the veterinary interest species classification, geese belong to the minor food-producing species (Toutain et al., 2010). Geese were domesticated a long time ago for their eggs, meat and feathers (Heikkinen, 2017; Honka et al., 2018). Waterfowls' meat and eggs have high nutritional quality and geese breeding is increasing all over the world, especially in Europe and Asia. Almost 60 different geese breeds exist, with many located in Eastern Europe (Buckland & Guy, 2002). The Bilgorajska goose (*Anser anser domesticus*), the subject of the present study in the scope of this Thesis, is a primitive breed from North-eastern Poland (Bilgoraj region) and is actively preserved because of its genetic significance (Ksiazkiewicz, 2006).

The health and productive performance of commercial geese is supported via modern pharmaceutical management and facilities, nutritional practices and genetic improvement. Infections, caused by pathogens such as *Mycoplasma* spp. or *Pseudomonas* spp., are common in geese, and other domesticated bird species (Stipkovits & Szathmary, 2012; Vos et al., 2011). These pathogens can infect eggs and destroy embryos. Levofloxacin shows activity against these and other pathogens. In the scope of this Thesis the levofloxacin pharmacokinetic study and tissue depletion study was performed, because geese, health is an important factor that constantly requires new protocols in pathogen prevention, control and treatment.

2 Methods

Three experimental studies were carried out in the scope of the current PhD Thesis. All results were published in separate original articles. Animal studies, bioanalytical laboratory and microbiological laboratory methods were used to complete this research.

The animal experiments were carried out in animal facilities of Latvia University of Life Sciences and Technologies Faculty of Veterinary Medicine (LBTU) (Jelgava, Latvia) and University of Life Sciences Department of Pharmacology, Toxicology and Environmental Protection (Lublin, Poland). The bioanalytical (liquid chromatography) sample analysis was performed at Rīga Stradiņš University Scientific Laboratory of Biochemistry (Riga, Latvia) and University of Pisa Department of Veterinary Sciences (Pisa, Italy). The microbiological assays were carried out at Rīga Stradiņš University Department of Biology and Microbiology (Riga, Latvia). Data analysis was performed at Rīga Stradiņš University Department of Pharmacology.

2.1 First study. Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

The study is described in the paper "Pharmacokinetic profiles of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits (*Oryctolagus cuniculus*)" by Andrejs Sitovs, Laura Voiko, Dmitrijs Kustovs, Liga Kovalcuka, Dace Bandere, Santa Purvina and Mario Giorgi. Journal of Veterinary Science, 2020 Mar; 21(2):e32. doi: 10.4142/jvs.2020.21.e32.. PMID: 32233138; PMCID: PMC60 PMC7113567 24462. Paragraphs 1.7, 2.1, 2.4, 3, 4.1 and 5.1 reference the aforementioned article.

2.1.1 Animals

Six cross-bred female rabbits (*Oryctolagus cuniculus*) (body mass 4.21 ± 0.74 kg), 6 months of age at the beginning of the study, were obtained from the animal facility of the Clinical Institute, Faculty of Veterinary Medicine, LBTU. Animals were determined to be healthy based on clinical examination, complete blood analysis, and complete ocular examination including biomicroscopy, indirect ophthalmoscopy, and tonometry. Animals received no drug treatment before the study and were allowed to acclimate in their cages for 7 days before the beginning of the study. Rabbits were housed individually in cages under 12-h light/12-h dark cycle with *ad libitum* access to drinking water and hay. Animals were fed standard pelleted food once daily (Purina Professional Rabbit Feed, Purina, USA). The room temperature was maintained at 20°C. Before the study, animals were randomly divided into 3 groups of 2 using research randomiser software. Identifying numbers were placed on each of the animal cages. Animals were weighed immediately before the beginning of the study and before every drug administration period.

2.1.2 Chemicals and reagents

Analytical standard (purity > 98 %) levofloxacin and enrofloxacin (used as the internal standard) and tetraethylammonium chloride were purchased from Sigma-Aldrich (USA). Acetonitrile, methanol, sodium dihydrogen phosphate, sodium hydrogen phosphate, chloroform, and isopropanol were of high-performance liquid chromatography grade. A levofloxacin solution (Levoflox 500 mg/100 mL; Claris, India) was used for administration to the animals.

2.1.3 Experimental design and sample collection

A 3-phase, 3-treatment cross-over study design was applied. The levofloxacin solution was administered as a single dose of 5 mg/kg body weight. In each phase, doses were administered as follows: IV route – as a 1 min bolus into the marginal ear vein; IM route — half of the dose was administered to each of the *musculus biceps femoris* consecutively (half dose used to avoid muscle damage due to large volume of solution to be administered); SC route — administered as an injection in the back of the neck region. A fourteen-day washout period was applied, allowing animals to fully clear the drug and to recover from stress related to the experimental procedures. Animal groups for levofloxacin administration were rotated until all 3 phases of the study were completed. For each phase, a sterile 24G catheter was placed in the central ear artery (for blood collection) and a second one into the marginal ear vein (for IV drug administration) prior to drug administration on the day of commencement of the experiment. The venous catheter was removed immediately after IV drug administration while the arterial one remained until blood collection at 10 hours post-administration. Catheters were flushed with heparin containing saline after blood collection, and before any blood collection, the first 0.3 mL of blood were discharged. Blood samples (approximately 0.5 mL) were collected immediately before levofloxacin administration and at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 hours post-administration. Blood samples at 24 and 48 hours were collected by syringe from the jugular vein. Collected blood was immediately transferred to lithium-heparin containing test tubes, centrifuged at 1000 × g for 10 min, and the plasma harvested and stored at −20°C until analysis. At 0.5, 2, 4, 8 and 10 hours additional 0.5 mL of blood was collected in a test tube without anticoagulant, left at room temperature to coagulate and serum was harvested and stored at −80°C for the pharmacodynamic study.

2.1.4 Tear fluid collection and analysis

Tear fluid evaluations included tests of tear production and tear film osmolarity. All evaluations were conducted the day before levofloxacin administration to obtain baseline values, and then at 1, 4, 8, 10, 24, and 48 hours after each levofloxacin administration. Schirmer

Tear Test (STT) values for tear production were obtained with standardised sterile Schirmer Tear Test I (Eickemeyer, Germany) tips that were inserted under the lower lateral eyelid margin for 1 min. The length of the wet section of the STT tip was immediately measured in millimetres (mm/min). Immediately after the STT result was obtained, STT strips were placed into 1.5 mL polypropylene vials and held at −20°C for further quantification of levofloxacin in the lacrimal fluid. Tear production was also evaluated by applying I-TEAR TEST strips (I-MED Pharma Inc., Canada) into both eyes at the same period post levofloxacin solution administration as that for the STT-based evaluations. A strip was applied to the central lower lid tear meniscus without touching the cornea or conjunctiva in accordance with the manufacturer's instructions. The number of millimetres on the strip reached in 5 seconds was obtained (unit: mm/5 sec). Tear film osmolarity was assessed by applying the I-PEN VET device (I-MED Pharma Inc., Canada) immediately after the tear production tests were performed. The I-PEN VET sensor was applied to the palpebral conjunctiva until a sound signal, indicating the end of the measurement, was heard (unit: mOsms/L).

2.1.5 Plasma chromatographic analysis

Levofloxacin concentrations in plasma samples were assessed using a Waters Acquity H Class Ultra Performance Liquid Chromatography system equipped with a fluorescence detector (Waters Corporation, USA). The chromatographic analytical method and the sample extraction procedure were based on those previously described (Lee et al., 2017). Briefly, to 200 µL of plasma, 100 µL of 10 µg/mL internal standard solution in methanol, 800 µL of phosphate buffer solution (pH = 7.0), and 4 mL of chloroform: isopropanol (5:1 v/v) were added. The mixture was shaken by a vertical rotating device (Biosan Bio-RS 24, Latvia) at 30 rotations per minute for 20 min, and then centrifuged at 3,000 × g for 10 minutes at 4°C. Three millilitres of the lower organic layer was transferred into a clean polypropylene tube and evaporated to dryness under a nitrogen stream at 40°C. The dry residue was reconstituted with 200 µL of the mobile phase. One microliter of the resultant solution was injected into the chromatographic system. The chromatographic column used was a Waters Acquity C18 BEH 2.1 × 75 mm with a 1.7 µm particle size (Waters Corporation). The column temperature was maintained at 35°C. The mobile phase was 83 % 0.02 M potassium dihydrogen phosphate solution with 0.012 M tetraethylammonium chloride (pH = 2.5) and 17 % acetonitrile. The isocratic flow rate was 0.3 mL/min. The fluorescence detector wavelengths were set to 295 nm excitation and 420 nm emission. The sample run time was 5 minutes.

2.1.6 Chromatographic method validation

Drug-free rabbit plasma was used for both standard curve construction and quality control method validation in accordance with the Guideline on Bioanalytical Method Validation EMEA/CHMP/EWP/192217/2009 (EMA, 2018a). Drug-free pooled plasma was harvested from all 6 experimental rabbits (2 mL of blood collected) immediately before the beginning of the first phase of the experiment but after the catheters had been placed. The calibration curve was linear from 0.01 to 10 μg/mL ($R^2 > 0.999$). The levofloxacin recovery from plasma was 96 % ± 3.5 %. The lower limit of quantification was 0.01 μg/mL. Five level standards of levofloxacin quality controls of 0.01, 0.025, 0.05, 0.5, and 5 μg/mL.

2.1.7 Pharmacokinetic analysis

Individual pharmacokinetic parameters were estimated for every animal after treatment using all 3 administration routes. Estimation was performed using non-compartmental analysis and based on visual inspection of the obtained graph (ThothPro Version 1.6.66, Poland). The linear trapezoidal interpolation method was used to calculate the AUC after IV administration, whereas the linear up/log down method was used for the IM and SC routes of administration. At least 3 of the last points of the elimination phase of the plasma vs. time curve were used to calculate the elimination constant. The C_{max}, and t_{max} were obtained from the data. The bioavailability (F %) was calculated for every single subject as F % = (AUC_{IM} or $_{SC}/AUC_{IV}$) × 100, and the mean absorption time (MAT) as MAT = MRT_{IM} or $_{SC} - MRT_{IV}$. Numerical differences of individual AUC_{0-last} values were lower than 20 % of AUC_{0-inf}, and the R^2 of the terminal phase regression line was > 0.85. Extraction ratio (E %) after IV administration was calculated using the clearance value after IV administration and the cardiac output value (i.e. E % = clearance/cardiac output ×100), where cardiac output = 180 × body weight$^{-0.19}$ (Toutain & Bousquet-Mélou, 2004b).

2.1.8 Pharmacokinetic-pharmacodynamic index

Because the levofloxacin concentrations were below the LOQ at 24 hours, in order to predict the AUC_{24} and to calculate the pharmacokinetic-pharmacodynamic surrogates, a dose 5 times that administered was modelled. The levofloxacin concentration values for all sampled times from 0.083 hours to 10 hours post-administration were multiplied by 5. Applying the superposition principle and assuming the same first-order kinetics (Gabrielsson & Weiner, 2001), approximate values of the concentration at 24 hours post-administration were calculated for each rabbit for all 3 routes of administration. The non-compartmental pharmacokinetic analysis was re-run to obtain an AUC_{24} value from this adjusted data, and the pharmacokinetic-pharmacodynamic surrogate AUC_{24}/MIC was calculated. Since fluoroquinolones produce a

concentration-dependent antimicrobial effect over time (Brown, 1996), a target AUC_{24}/MIC ratio for fluoroquinolones of 72 was used (Madsen et al., 2019).

2.1.9 Drug accumulation prediction

A prediction based on a single administration was used to evaluate the possible accumulation ratio (R) at 12 h dosing intervals (τ). The following formula was used (Toutain & Bousquet-Mélou, 2004c):

$$R = \frac{1}{[1-(0.5)^{\frac{\tau}{t_{1/2}}}]} \qquad (1)$$

where τ is the dosing interval and $t_{½}$ is the half-life of elimination.

2.1.10 MIC breakpoints prediction

Based on the equation AUC_{24}/MIC > 72, the antimicrobial activity breakpoint for the theoretically computed dose of 25 mg/kg for rabbits, a MIC < AUC_{24}/72 was assumed to be effective (Madsen et al., 2019). The AUC was expressed in terms of the unbound drug; levofloxacin was previously reported to be 25 % bound to plasma proteins in rabbits (Destache et al., 2001).

2.1.11 Theoretical effective daily dose calculation

As fluoroquinolones are antimicrobials that possess concentration/time-dependent effects, a theoretical optimal daily dosage was calculated for all 3 routes of administration based on the following formula (Toutain et al., 2002):

$$Dose\ per\ day = \frac{\frac{AUC_{24}}{MIC} \times \mathrm{MIC} \times \mathrm{Cl}}{f_u \times F} \times 24 \qquad (2)$$

where AUC_{24}/MIC is the ratio for optimal efficacy (= 72), Cl = clearance, f_u = free fraction of drug in plasma (= 0.75) and F = bioavailability (considered 1 if complete).

2.2 Second study. Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

The study is described in the paper "Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese" by Irene Sartini, Beata Łebkowska-Wieruszewska, Andrejs Sitovs, Andrzej Lisowski, Amnart Poapolathep and Mario Giorgi. British Poultry Science, 2021 Apr;62(2): 193–198. doi:

10.1080/00071668.2020.1842855. Epub 2020 Nov 18. PMID: 33121260. Paragraphs 1.8, 2.2, 2.4, 3, 4.2 and 5.2 reference the aforementioned article.

2.2.1 Animals

Geese were supplied by a local farm (Majątek Rutka, Puchaczów, Poland). Their health status was evaluated based on a complete physical examination by a veterinarian before the beginning of the study, and through daily observation of behaviour and appetite. Geese were acclimatised for one week in their new environment before the beginning of the trial, and a ring with an identity code was applied to the left leg for easy identification. Birds were housed in a 60 m^2 enclosed area with an indoor shelter of 8 m^2. Animals were allowed to graze freely during the day and were fed a balanced, drug-free pelleted diet (Purina Duck Feed pellets, Purina Animal Nutrition, Gray Summit, MO, USA) twice a day and water was supplied *ad libitum*. No pharmacological treatment was received by the birds before the experiment.

2.2.2 Chemicals and reagents

Levofloxacin and the internal standard (IS) enrofloxacin powder with a standard purity of 99.0 % were purchased from Sigma-Aldrich (Milan, Italy). High performance liquid chromatography (HPLC)-grade acetonitrile, methanol, trichloromethane and isopropanol were procured from Merck (Kenilworth, NJ, USA). Tetraethylamine was obtained from Sigma-Aldrich (St Louis, MI, US). Orthophosphoric acid, sodium dihydrogen phosphate and potassium hydrogen phosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionised water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

2.2.3 Experimental design and sample collection

The study consisted of two parts – pharmacokinetic trial and a tissue depletion trial. The pharmacokinetic trial involved 16 healthy male Bilgorajska geese (body weight (BW), 3.4–4.9 kg; age, 3–4 years) which were randomly divided into two sub-groups (n = 8/group). Sub-group 1 received a single IV dose (2 mg/kg) of levofloxacin (levofloxacin TEVA 5 mg/mL; Teva Pharmaceutical, Hungary) into the left brachial vein using a sterile 26-gauge 1.75 cm needle. The geese in sub-group 2 were given a single oral dose (5 mg/kg) of levofloxacin. The oral doses were prepared by grinding, homogenising, and partitioning the marketed drug (Levofloxacin ACCORD 250 mg/tablet; Accord Healthcare Limited, UK) and dosed relative to the BW of each bird. The correct weight of the solid formulation was dissolved in water and administered via crop gavage using a rounded tip metal catheter 3 h after being fed. Blood samples (1 mL) were collected in vacutainer lithium heparin tubes (BD, Vaud, Switzerland)

from a 24-gauge catheter inserted immediately before the experiment in the right brachial vein at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 10, 24, 34, and 48 hours after IV and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h after the last drug administration after per os (PO) treatment. After 12 hours, the catheter was removed, and blood was collected from the left brachial vein directly with a 24-gauge syringe. The catheter was cleaned by flushing with 1 mL of 0.9 % saline with the addition of 10 IU/mL heparin at each collection timepoint. For each blood collection, the first 0.2 mL of blood was discarded. Tubes were centrifuged at 1500 x g and the harvested plasma was stored at −20°C until analysis within 30 days of collection.

The tissue depletion trial involved 10 geese which were given an oral dose (5 mg/kg) of levofloxacin, as described for sub-group 2. Two animals were humanely killed by stunning and exsanguination at 6, 10, 24, 34 and 48 hours after treatment. Approximately 4 g of muscle, heart, liver, lung and kidney were collected and stored at −20°C until further analysis.

2.2.4 Plasma and tissue extraction procedure and chromatographic analysis

An aliquot (0.2 mL) of plasma was added to 0.1 mL of IS (0.1 μg/mL) solution in methanol and 0.8 mL of 0.1 M phosphate buffer at pH 7.1. After the addition of 6 mL of a mixture of trichloromethane and isopropanol (5:1 v/v %), the samples were shaken at 60 oscillations/minute for 10 minutes and centrifuged at 4000 x g for 5 minutes. Then 5 mL of the organic layer was transferred into a clean tube and dried at 40°C under a nitrogen stream. The residue was dissolved in 0.2 mL of mobile phase, vortexed and an aliquot (50 μL) was injected on to the HPLC system. Liver, kidney, lung, heart and muscle samples were thawed and immediately dissected into small pieces and an aliquot of 1 g per tissue was placed into 5 mL plastic tubes containing 3 mL of homogenisation solution (0.1 M phosphate buffer at pH 7.1). The suspension was homogenised for approximately 40 seconds and then 0.2 mL were processed, as described for the plasma samples.

The HPLC was an LC system (Jasco, Japan) consisting of a high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus), auto sampler (model AS 950), and Peltier system (model CO-4062). The injection loop volume was set at 50 μL. Data was processed using the CromNav 2.0 software (Jasco, Inc., Japan). The chromatographic separation assay, modified from the method reported in the literature (Lee et al., 2017), was performed using a Gemini analytical column (250 × 4.6 mm inner diameter, 5 μm particle size, Phenomenex, Torrance, California, USA) at 15 °C. The mobile phase consisted of acetonitrile: aqueous solution (20:80 v/v %) at a flow rate of 1 mL/minute. The aqueous solution consisted of potassium dihydrogenphosphate (0.02 M), phosphoric acid (0.006 M) and tetraethyl amine

(0.012 M) in water (pH = 4.0). Excitation and emission wavelengths were set at 295 and 490 nm, respectively.

2.2.5 Chromatographic method validation

The quantitative HPLC method was fully validated for each tissue (liver, kidney, lung, heart and muscle) and plasma in terms of linearity, intra-day and inter-day precision, recovery, limits of detection (LOD) and quantification (LOQ), according to the EMA guidelines (EMA, 2018a). Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n=3) of samples from the control geese spiked with levofloxacin at concentrations of 0.005, 0.01, 0.1, 0.5, 1, 5 μg/mL. The intra- and inter-day precision was calculated after analysis of six plasma resamples spiked with levofloxacin at three different concentrations (0.005, 0.1 and 5 μg/mL) with the same instrument and the same operator on the same and on different days, respectively. Precision was calculated and expressed as the coefficient of variation (CV %). The extraction recovery experiment was carried out by analysing samples spiked with the same concentration (0.005, 0.1 and 5 μg/mL) by comparing the response (measured as area) of high, middle, low standards and the IS spiked into blank goose plasma (control), to the response of equivalent standards. Recovery was expressed as mean ± standard deviation (SD). The LOD was estimated as the plasma and tissue drug concentrations that produced a signal to noise ratio of 3 and LOQ was determined as the lowest plasma concentration that produced a signal to noise ratio of 10.

2.2.6 Pharmacokinetic analysis

Levofloxacin plasma concentration was modelled for each subject using a non-compartmental model using ThothPro 4.3.0 v software (www. thothpro.com, Gdansk, Poland). The C_{max} and time to reach the C_{max} (t_{max}) were determined directly from the concentration vs time curves. The elimination half-life ($t_{1/2}\lambda_z$) was calculated using least squares regression analysis of the concentration-time curve, and the AUC was calculated by linear log trapezoidal and the linear-up log-down rule was applied to the final concentration-time points for both IV and PO administration, respectively. From these values, the volume of distribution at steady state (V_{ss} = dose x $AUMC/AUC^2$), mean residence time (MRT = AUMC/AUC), and systemic clearance (Cl=dose/AUC) were calculated. Pharmacokinetic estimates were calculated only if the individual value of $AUC_{rest\%}$ was lower than 20% % of $AUC_{0\text{-}inf}$ and the square of coefficient of determination (R^2) of the terminal phase regression line was >0.85.

Absolute oral bioavailability (F %) was calculated using the following formula:

$$F\ (\%) = \frac{AUC_{P/O\ individual} \times Dose_{I/V}}{AUC_{I/V\ average} \times Dose_{P/O}} \times 100 \qquad (3)$$

A naïve pooled-data approach, using a non-compartmental analysis (Pouplin et al., 2016), was used to calculate the pharmacokinetic parameters for levofloxacin in all tissue samples. The penetration of levofloxacin into each tissue was determined by comparing the AUC ratios between tissues and plasma ($AUC_{tissue}/AUC_{plasma}$) after PO administration (Sartini et al., 2020). Levofloxacin concentrations in the selected tissues were used to calculate preliminary withdrawal times using the software WT 1.4, developed by the European Medicines Agency (EMA, 2018b). The withdrawal time was established as being the time when the upper-one sided tolerance limit (99 %) with 95 % confidence interval (CI) was below the maximum residue limit of 0.1 μg/g levofloxacin, which reflected the MRL for fluoroquinolones in poultry liver (EMA, 1997, 1999, 2002).

2.3 Third study. *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*)

The study is described in the paper "*In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*) – A preliminary study" by Andrejs Sitovs, Ingus Skadins, Santa Purvina and Dace Bandere. Journal of Veterinary Pharmacology and Therapeutics, 2023 Apr 15; Online ahead of print. doi: 10.1111/jvp.13383. PMID: 37060264. Paragraphs 1.7, 2.3, 2.4, 4.3 and 5.3 reference the aforementioned article.

2.3.1 Bacterial isolates

This study included *P. multocida* clinical isolates (n = 10), *E. coli* isolates (n = 5) and commercially available *E. coli* ATCC 25922 (ATCC, USA) as a reference strain. All *E. coli* isolates were collected from rectal swabs of clinically healthy rabbits that did not previously receive any treatment. Health status was verified by the veterinarian, based on the physical examination and complete blood analysis. Rabbits were housed on a farm near Riga, Latvia. Rectal swabs from were obtained using TRANSWAB® Gel Amies Plain (MWE, UK) with gel media. Within the same day, the samples were transported to the laboratory of microbiology at Rīga Stradiņš University. Swabs were cultured on McConkey agar and identified with VITEK2 Compact system (bio- Mérieux, France). One *E. coli* isolate from one rabbit was selected. Isolates were considered part of commensal flora and not pathogenic. All *P. multocida* isolates

were from rabbits with clinical rhinitis and/or pneumonia. Six *P. multocida* isolates were provided by the Faculty of Veterinary Medicine, Latvia University of Life Sciences and Technologies (Jelgava, Latvia), and 4 isolates were provided by the Institute of Food Safety Animal Health and Environment BIOR (Riga, Latvia). One *P. multocida* isolate from one rabbit was used in this study.

2.3.2 Determination of minimum inhibitory and minimum bactericidal concentrations in broth and serum

Minimum inhibitory concentration values were determined using the microdilution method according to the CLSI guidelines M100 (CLSI, 2018a, 2018b). Levofloxacin standard (>99 %) was purchased from Sigma-Aldrich. Levofloxacin stock solution (5120 μg/ mL) was prepared in Milli-Q ultra-purified water (Millipore, USA) with the addition of 0.1 M NaOH and further diluted to working concentrations with cation-adjusted Mueller Hinton broth (MHB) or in commercially available drug-free sterile rabbit serum (Biowest, France), respectively. *Escherichia coli* MIC and MBC were detected in MHB and serum. Pasteurella multocida MIC and MBC were determined in MHB with the addition of 5 % defibrinated sheep blood (bioTRADING Benelux B.V., France) and in serum. After the overnight growth on agar plates, colonies were suspended in MHB to reach the same turbidity as the McFarland turbidity standard of 0.5. Each *E. coli* culture was diluted 1:100 in MHB to obtain a bacterial count of approximately 10^6 colony-forming units per millilitre (CFU/mL); each *P. multocida* culture was diluted 1:100 in MHB supplemented with 5 % defibrinated sheep blood. Levofloxacin 128 μg/mL working solutions were prepared in MHB and in serum. Final incubation for 24 hours at 37°C was performed with levofloxacin serial dilutions from 64 to 0.004 μg/mL in both media in the presence of 5 x 10^5 CFU/mL of bacteria. After the incubation, *E. coli*-containing microdilution plates were read at 600 nm using Infinite F50 Plus reader (Tecan, Switzerland). MIC was reported as the lowest levofloxacin concentration, which showed no turbidity in the microdilution tray wells. For *P. multocida* in MHB with blood, MIC was reported as the lowest concentration where no colour change from red to brown was visually observed. To determine the MBC, 10 μL of the content of wells showing no bacterial growth was transferred to plates, containing Tryptic Soy Agar (TSA) for *E. coli* and TSA supplemented with 5 % defibrinated sheep blood for *P. multocida*. After incubation for 24 hours at 37°C, colonies were counted. The limit of detection was 100 CFU/mL. The lowest concentration showing no bacterial growth was reported as MBC. Reference culture *E. coli* ATCC 25922 MIC and MBC values were determined on MHB only. Experiments were performed in triplicate.

2.3.3 Levofloxacin serum samples for *ex vivo* bacterial killing curve evaluation

Serum samples containing levofloxacin at known concentrations were obtained from our rabbit levofloxacin pharmacokinetic profile study. There, after each drug administration, serum samples for *ex vivo* study were obtained after 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 hours. Pooled serum samples from experimental rabbits (3 mL) were used for the present study. Levofloxacin concentrations in pooled serum samples were determined prior to the time-killing study with a validated HPLC method (Sitovs et al., 2020).

2.3.4 In vitro bacterial killing curves for Pasteurella multocida and Escherichia coli

One isolate of *P. multocida* and one isolate of *E. coli* were chosen to be used in the bacterial time-killing curve study. The bacterial killing curve study protocol was based on the method described in the literature (Lee et al., 2017). Levofloxacin solutions in drug-free rabbit serum were prepared at concentrations relative to the MIC in the serum of the bacterial isolate. For *P. multocida*, concentrations were 0.00 μg/mL (control), 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 times the MIC and for *E. coli* concentrations were 0.00 μg/mL (control), 0.5, 1, 2, 4, 8, 16 and 32 times the MIC. For each bacterial isolate, 8 colonies from overnight growth on agar plates were added to 9 mL of MHB and incubated for 20 hours at 37°C in presence of 5 % CO_2. Ten microlitres of broth culture were added to 1 mL of levofloxacin solutions in serum in order to reach the concentration of approximately 1.6×10^6 CFU/ mL for P. multocida isolate and 2×10^7 CFU/mL E. coli. Samples were incubated for 24 hours at 37°C in an orbital shaker; 20 μL from all samples were withdrawn at 3, 6 and 24 hours of incubation. Prior to withdrawal, samples were vortexed. Dilutions ranging from 10^{-1} to 10^{-8} in sterile 0.9 % saline were prepared to count the CFU. A 10 μL volume of each saline dilution was inoculated on a TSA plate and incubated for 16 hours. TSA plates for *P. multocida* samples were supplemented with 5 % defibrinated sheep blood. CFU were counted and the limit of detection was 100 CFU/mL. The count of bacteria in the initial inoculum was approved with the same dilution in the sterile saline method. All experiments were performed in triplicate.

2.3.5 *Ex vivo* bacterial killing curves for *Escherichia coli* and *Pasteurella multocida*

The same *P. multocida* and *E. coli* isolates, as for the *in vitro* bacterial killing study, were used in the *ex vivo* study. The study protocol was almost identical, to the *in vitro* bacterial killing. The difference was that instead of levofloxacin dilutions in antibiotic-free rabbit serum, we used serum samples obtained from rabbits that received 5 mg/kg of levofloxacin parenterally. Pooled serum samples collected at 0, 0.5, 1, 2, 4, 8 and 10 hours after administration contained 0.00, 3.26, 2.64, 1.48, 0.58, 0.13 and 0.07 μg/mL for IM and 0.00,

2.59, 2.70, 1.91, 0.75, 0.14 and 0.08 μg/mL for SC routes of administration, respectively. All experiments were performed in triplicate.

2.3.6 Pharmacodynamic modelling and daily dose calculation

To determine AUC_{24}/MIC ratios, each *in vitro* levofloxacin concentration was multiplied by 24 (period of incubation) and then divided by the MIC value of each bacterial isolate tested, respectively. The relationship between *in vitro* AUC_{24}/MIC and $\log_{10}$ difference in bacterial count from the initial inoculum to the bacterial count after 24 hours of incubation for serum was evaluated by using the sigmoid inhibitory I_{max} model in Phoenix WinNonlin (Certara, USA). Akaike's Information Criterion was applied to determine the goodness of fit. The model is described with the following equation:

$$E = E_0 - \frac{I_{max} \times C^{\gamma}}{C^{\gamma} + IC_{50}^{\gamma}} \qquad (4)$$

E – antibacterial effect of levofloxacin; I_{max} – difference between $\log_{10}$ difference in bacterial count between 0 and 24 hours in the control sample ($\log E_0$) and the $\log_{10}$ difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached; E_0 – $\log_{10}$ difference in the bacterial count from 0 to 24 hours of incubation in the control sample, antibiotic-free; IC_{50} is the AUC_{24}/MIC producing 50 % of the maximal antibacterial effect; C is the AUC_{24}/MIC in the effect compartment (serum); γ–the Hill coefficient which characterises the slope of the AUC_{24}/MIC response curve.

The antibacterial activity of levofloxacin against both bacteria species in this study was assessed by calculation of AUC_{24}/MIC values required for bacteriostatic, bactericidal effects and bacterial elimination. AUC_{24}/MIC for bacteriostatic effect was calculated using E = 0, that is, no change in bacterial counts after the incubation for 24 h with levofloxacin. AUC_{24}/MIC for bactericidal effect was calculated using E = −3, that is, bacterial counts reduction by 99.9 % after the incubation for 24 hours with levofloxacin. AUC_{24}/MIC for bacterial elimination effect was calculated using the lowest E value when the maximal antibacterial effect was reached, that is, bacterial count reduction to the limit of quantification (100 CFU/mL) after the incubation for 24 hours with levofloxacin. Obtained from pharmacokinetic–pharmacodynamic integration, antibacterial effects AUC_{24}/MIC values were used to calculate optimal doses for three effect levels – bacteriostatic, bactericidal and bacterial elimination. The following formula (McKellar et al., 2004) was used:

$$Dose\ per\ day = \frac{\frac{AUC_{24}}{MIC} \times \mathrm{MIC} \times \mathrm{Cl}}{f_u \times F} \times 24 \qquad (5)$$

AUC_{24}/MIC are ratios for bacteriostatic, bactericidal and bacterial elimination effects, MIC is minimum inhibitory concentration in serum, Cl is clearance, F is bioavailability, and f_u is a free fraction of levofloxacin in plasma. The following values were used, Cl = 0.6 mL/g/h and F = 1 (Sitovs et al., 2020). Levofloxacin protein binding in rabbit plasma was 25 %, thus, f_u = 0.75 (Destache et al., 2001).

2.4 Ethics statement

For the levofloxacin pharmacokinetics and pharmacodynamics studies in rabbits, the experimental protocol was approved by the Animal Ethics Committee of the Republic of Latvia Food and Veterinary Service (Permission 025564). The study was performed according to the guideline for the care and use of laboratory animals in accordance with the European law (2010/63/UE). For the levofloxacin pharmacokinetics and tissue depletion study in geese, the experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Life Sciences (Lublin, Poland) and carried out in accordance with the European law (2010/63/UE).

3 Statistical Analysis

In the pharmacokinetics study after intravenous, intramuscular and subcutaneous administration of levofloxacin to rabbits, the statistical analysis of pharmacokinetic parameters was performed using SPSS (version 21.0; IBM Corporation, USA). Most statistical parameters are reported as mean ± standard deviation (SD) values. The exceptions are for plasma half-lives (harmonic means were calculated) and t_{max} (median values are reported). The normality of the data was assessed using the Shapiro-Wilk test. Paired t-tests were used to compare the statistical differences for pharmacokinetic parameters with normal data distributions in different administration groups. Where data did not have a normal distribution (e.g. V_{area}/F after IM or SC administration), the Wilcoxon test was applied. The p values lower than 0.05 were considered to indicate statistical significance. In the pharmacokinetics and tissue depletion study in geese, the pharmacokinetic parameters were checked for normal distribution by Shapiro–Wilk test and mean pharmacokinetic values were compared between the two routes of administration using unpaired t-tests using GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The p values lower than 0.05 were considered to indicate statistical significance.

4 Results

4.1 First study. Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

4.1.1 Animals

All 6 animals received levofloxacin via IV or IM routes; however, only 4 completed the SC administration. In the third phase of the cross-over study, 2 animals were excluded—one animal was excluded because of the inability to fix the catheter in either ear artery. The other animal suffered cramps post IV administration of levofloxacin and died within 48 hours post-administration. Post-mortem examination of this animal showed no respiratory tract, kidney, gastrointestinal tract, or liver abnormalities.

4.1.2 Pharmacokinetic parameters

For all 3 administration routes, the drug was quantifiable in plasma for up to 10 hours post-administration of 5 mg/kg. The semilogarithmic plots of mean levofloxacin plasma concentrations (± SD) after the 5 mg/kg single dose via all 3 routes of administration are presented in Figure 4.1. The mean values of pharmacokinetics parameters obtained (± SD) are reported in Table 4.1. The average AUC_{0-last} values were 9.03 (± 2.66), 9.07 (± 1.80) and 9.28 (± 1.56) µg×h/mL after IV, IM, and SC administration, respectively. Maximum plasma concentration reached 3.33 (± 0.39) and 2.91 (± 0.56) µg/mL after IM and SC administrations, respectively. The mean extraction ratio after 5 mg/kg IV administration was 7.2 % ± 2.1 %.

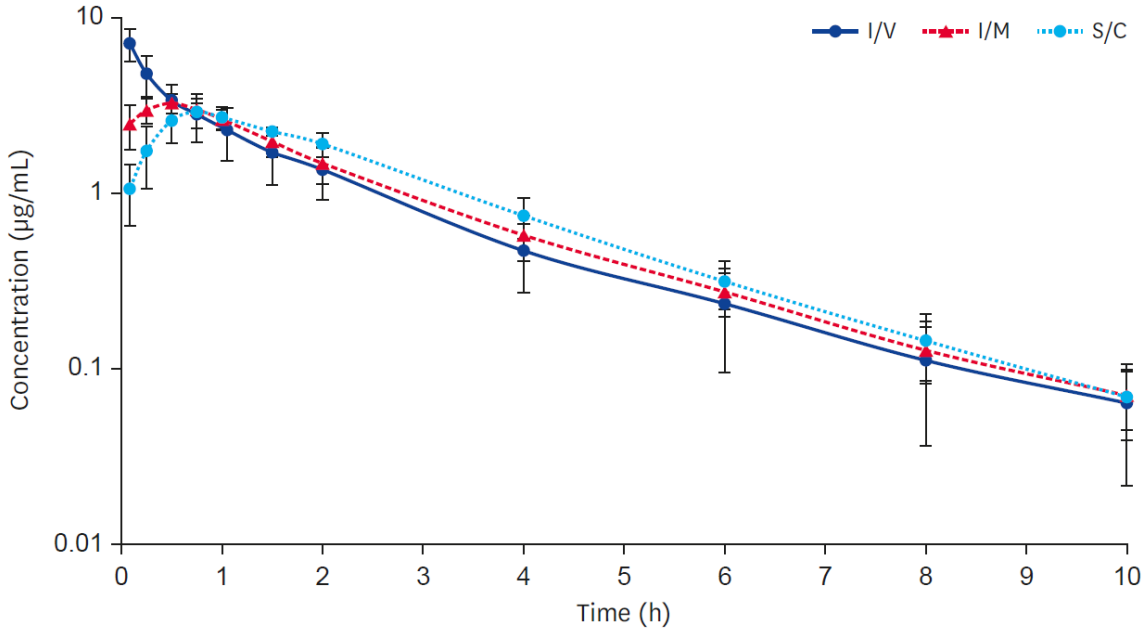

Figure 4.1 **Semilogarithmic plots of average levofloxacin plasma concentrations in rabbits (error bars represent standard deviations) after IV (n = 6), IM (n = 6), and SC (n = 4) levofloxacin administration of 5 mg/kg bodyweight**

IV, intravenous; IM, intramuscular; SC, subcutaneous.

Table 4.1

Mean (± SD) pharmacokinetic parameters of levofloxacin in plasma following IV, IM or SC administration to rabbits at a dose of 5 mg/kg bodyweight

PK parameter	Units	IV (n=6)	IM (n=6)	SC (n=4)
$AUC_{0\text{-last}}$	µg×h/mL	9.03±2.66	9.07±1.80	9.28±1.56
$AUC_{0\text{-inf}}$	µg×h/mL	9.08±2.64	9.07±1.80	9.31±1.50
$AUMC_{0\text{-last}}$	µg×h×h/mL	22.93±12.46	37.87±18.35•	36.62±17.35
$AUMC_{0\text{-inf}}$	µg×h×h/mL	23.64±12.17	37.89±18.34•	36.98±16.82
C_{max}	µg/mL	N/A	3.33±0.39	2.91±0.56
C_{first}	µg/mL	7.13±1.47	N/A	N/A
t_{max} MEDIAN	h	N/A	0.50 (0.08–0.75)	0.75
$t_{1/2}\lambda_z$ HM	h	2.06±0.18	2.01±0.24	1.80±0.14
λ_z	1/h	0.34±0.03	0.34±0.04	0.39±0.03
$MRT_{0\text{-last}}$ HM	h	2.19±0.83	3.75±1.16•	3.44±1.31
$MRT_{0\text{-inf}}$ HM	h	2.27±0.80	3.75±1.16•	3.52±1.25
MAT HM	h	N/A	1.29±0.61	0.45±1.47
Cl	mL/g×h	0.60±0.18	N/A	N/A
Cl/F	mL/g×h	N/A	0.57±0.11	0.55±0.10
V_{ss}	mL/g	1.37±0.39	N/A	N/A
V_{area}/F	mL/g	N/A	1.66±0.34	1.42±0.18
F	%	N/A	105.69±27.50	118.93±40.51

PK, pharmacokinetic; AUC_{0-last}, area under the plasma-concentration time curve from zero to the last quantified sampling point time; AUC_{0-inf}, area under the plasma-concentration time curve from zero extrapolated to infinity; $AUMC_{0-last}$, area under the first moment curve from zero to the last quantified sampling point time; $AUMC_{0-inf}$, area under the first moment curve from zero extrapolated to infinity; C_{max}, maximum plasma drug concentration; C_{first}, concentration at first sample collection point; t_{max}, time of the maximum plasma concentration; $t_{1/2}\lambda_z$, half-life of the elimination part of the curve; λ_z, slope of the elimination part of the curve; MRT_{0-last}, mean residence time from zero to the last quantified sampling point time; MRT_{0-inf}, mean residence time from zero extrapolated to infinity; MAT, mean absorption time; Cl, total plasma clearance; Cl/F, plasma clearance corrected to the bioavailability; V_{ss}, volume of distribution at steady-state; V_{area}/F, volume of distribution corrected to the bioavailability; n, number of experimental animals receiving levofloxacin via the corresponding route of administration; IV, intravenous; IM, intramuscular; SC, subcutaneous; N/A, not applicable; HM, harmonic mean. •Significantly different from IV administration ($p < 0.05$); †Range reported.

4.1.3 Pharmacokinetic-pharmacodynamic index

The *in silico* obtained AUC_{24} values for the theoretical dose of 25 mg/kg were 44.98 ± 12.54 mg × h/L for IV administration, 43.11 ± 6.85 mg × h/L for IM administration, and 43.62 ± 13.65 mg × h/L for SC administration. The levofloxacin accumulation ratio when administered twice daily (τ = 12 hours) was predicted to be 1.019 ± 0.006. To obtain the AUC_{24}/MIC of 72, considering that levofloxacin is 25 % bound to plasma proteins, it was calculated that 25 mg/kg of levofloxacin by IV administration would be effective against pathogens with a MIC < 0.47 μg/mL. In the case of IM and SC routes of administration, this dose would be effective against pathogens with a MIC < 0.45 μg/mL. Thus, an effective daily dose against pathogens with a MIC of 0.5 μg/mL was calculated for the IV administration to be 29 ± 8 mg/kg body weight.

4.1.4 Effects on tear quality

Average tear production observed with STT was 6.4 ± 3.1 mm/min and 7.0 ± 3.1 mm/min, for left and right eyes, respectively (no significant difference, p = 0.536). Absolute values varied from 2 to 14 mm/min. No significant changes in tear production were observed among all routes of drug administration within 48 hours. Strip meniscometry values, obtained by following the manufacturer's instructions, of 5 mm and higher are considered to indicate normal tear production while smaller values suggest decreased tear production. The average SM measurement results were normal, 6.9 ± 1.3 mm/5 sec and 6.3 ± 1.9 mm/5 sec, for the left and right eyes, respectively (no significant difference, p = 0.145). No significant changes in tear production after levofloxacin IV, IM, and SC administration were observed. Tear osmolarity was 324 ± 21 mOsms/L and 331 ± 22 mOsms/L for both eyes (right and left) prior to drug administration, and the difference was not significant (p = 0.255). Mean tear osmolarity decreased in all 3 routes of administration within 48 h after treatment. Changes in tear osmolarity up to 48 hours after levofloxacin administration are summarised in Figure 4.2. Another area of interest was the quantification of the levofloxacin level in tear fluid in order to evaluate the rationale of ocular infection treatment (conjunctival and corneal infection treatments may be affected by drug distribution in tears). However, the small volume of tear fluid harvested, and the limited sensitivity of the detection method used did not allow quantification of levofloxacin in rabbit tear fluid.

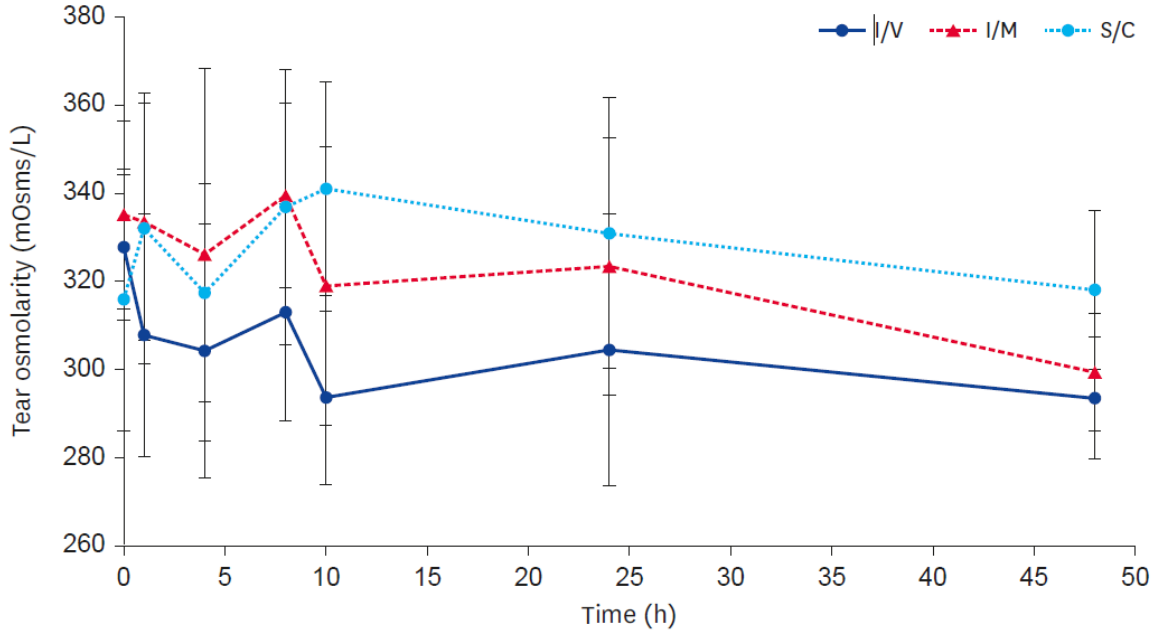

Figure 4.2 **Changes in tear osmolarity in rabbits after a single 5 mg/kg levofloxacin dose administered via IV (n = 6), IM (n = 6), or SC (n = 4) routes (mean values indicated; error bars represent standard deviation).**

IV, intravenous; IM, intramuscular; SC, subcutaneous

4.2 Second study. Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

4.2.1 Animals

The geese did not show any adverse effects during or after drug treatments. All animals received levofloxacin via IV or PO routes and all of them completed the study.

4.2.2 Analytical method validation

The validated analytical method showed a good linearity in the range of 0.005 – 5 µg/mL for every matrix considered in this study. The main results from the analytical method validation in plasma and all tissues selected are reported in Table 4.2.

Table 4.2

Levofloxacin HPLC analytical method validation results in plasma and goose tissues

Parameter	Unit	Plasma	Muscle	Heart	Liver	Lung	Kidney
Inter-day CV	%	5.6	6.1	5.9	6.0	8.9	7.2
Intra-day CV	%	6.9	10.9	9.6	7.4	10.6	9.9
Recovery	%	96 ± 5	94 ± 10	95 ± 8	98 ± 3	93 ± 8	91 ± 9
LOD	µg/mL	0.001	0.001	0.001	0.001	0.001	0.001
LOQ	µg/mL	0.005	0.005	0.005	0.005	0.005	0.005

4.2.3 Pharmacokinetic results

The semilogarithmic plasma concentration vs time curves after IV and PO administration of a single dose of levofloxacin at 2 mg/kg and 5 mg/kg, respectively are shown in Figure 4.3. Plasma levofloxacin concentrations were quantifiable up to 24 hours in birds administered intravenously, and up to 48 hours after PO treatment. The slope of the elimination phase appears to be similar for both routes of administration (Table 4.3).

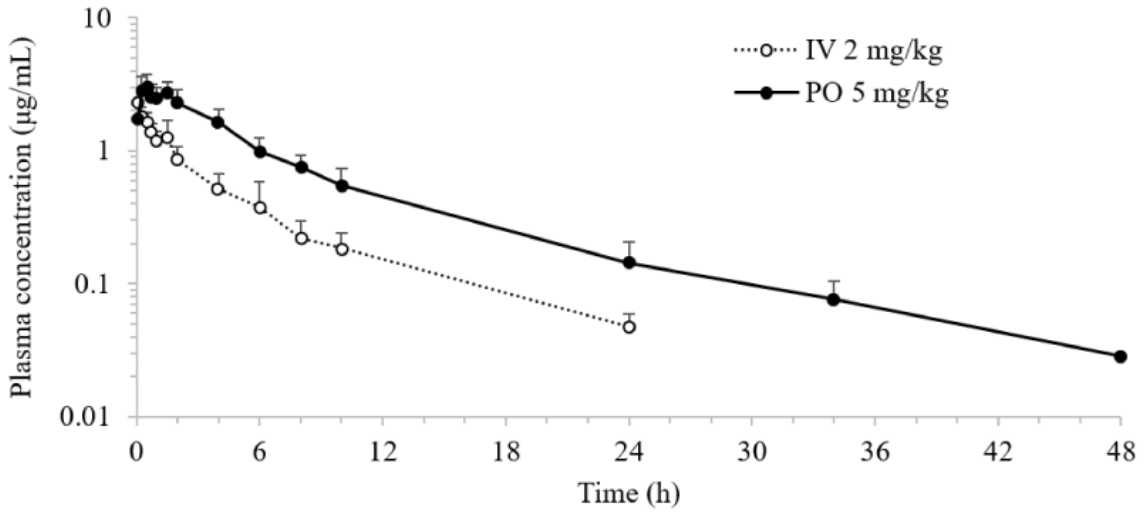

Figure 4.3 **Semilogarithmic plasma levofloxacin concentrations vs time curve following IV (–○–, n = 8) and PO (–●–, n = 8) administration to Bilgorajska geese at a dose of 2 mg/ kg BW and 5 mg/ kg BW, respectively.**

Table 4.3 shows the main pharmacokinetic parameters for levofloxacin in geese. Levofloxacin was absorbed rapidly after PO administration displaying a high bioavailability. The drug showed a moderate volume of distribution and a fast clearance. The half-life was not statistically different between the two routes of administration. If normalised for the dose, C_{max} and AUC were not statistically different between the two different administration methods ($p > 0.05$).

Table 4.3

Mean pharmacokinetic parameters of levofloxacin in plasma following IV administration to geese at a dose of 5 mg/kg and PO administration to geese at a dose of 2 mg/kg

		IV (2 mg/kg)		PO (5 mg/kg)	
Parameter	**Unit**	**Mean**	**SD**	**Mean**	**SD**
$AUC_{0\text{-last}}$	mg×h/L	7.59	1.77	17.24	4.86
$AUC_{0\text{-inf}}$	mg×h/L	8.11	1.76	19.37	4.18
$MRT_{0\text{-last}}$	h	5.12	0.37	5.71	2.48
$MRT_{0\text{-inf}}$	h	7.08	0.97	7.65	2.17
λ_z	1/h	0.10	0.02	0.12	0.05
$t_{1/2}\lambda_z$	h	7.39	1.21	6.60	2.46
V_{ss}	mL/g	1.40	0.28	N/A	N/A
Cl	mL/g×h	0.28	0.06	N/A	N/A
V_{ss}/F	mL/g	N/A	N/A	1.63	0.49
Cl/F	ml/g×h	N/A	N/A	0.31	0.09
C_{max}	µg/mL	N/A	N/A	3.20	0.65
t_{max}†	h	N/A	N/A	0.38	(0.25–1.5)
F	%	N/A	N/A	95.57	20.61

$AUC_{0\text{-last}}$, area under the curve from 0 hours to last time collected samples; $AUC_{0\text{- inf}}$, area under the curve from 0 hours to infinity; $MRT_{0\text{-last}}$, mean residence time from 0 hours to last time collected samples; $MRT_{0\text{-inf}}$, mean residence time from 0 hours to infinity; λ_z, terminal phase rate constant; $t_{1/2}\lambda_z$, terminal half-life; V_{ss},volume of distribution; Cl, plasma clearance; V_{ss}/F, volume of distribution normalised for F; Cl/F, plasma clearance normalised for F; C_{max} peak plasma concentration; t_{max}, time of peak concentration; F, bioavailability; † Median value and range; N/A, not applicable

4.2.4 Tissues residue analysis results

Results from tissue residue analysis are displayed in Figure 4.4 as semilogarithmic plots of tissue concentrations vs time curves. Drug residues were highest at 6 hours and decreased constantly, remaining over the LOQ up to 48 hours (last time-point of collection) in all selected tissues. Liver samples had the highest levofloxacin concentration, followed by kidney samples (Table 4.4).

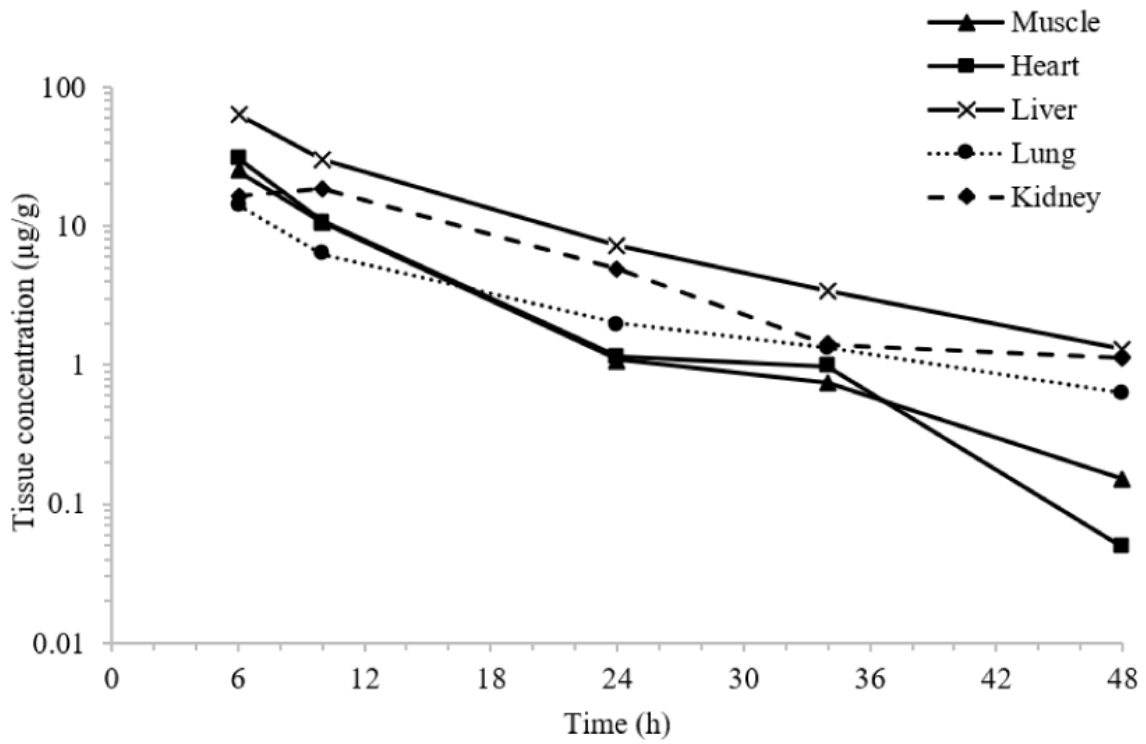

Figure 4.4 **Levofloxacin concentrations (logarithmic scale) in muscle, heart, liver, lung, and kidney following PO administration to Bilgorajska geese (n =2 /timepoint) at a dose of 5 mg/kg BW**

Table 4.4

Mean pharmacokinetic parameters, calculated by the naïve pooled-data approach for each tissue after PO administration to geese at a dose of 2 mg/kg

Parameter	Unit	Muscle	Heart	Liver	Lung	Kidney
$AUC_{0\text{-last}}$	µg×h/mL	218.72	249.8	687.94	165.26	329.51
$MRT_{0\text{-last}}$	h	10.41	9.94	12.56	14.31	13.58
$t_{1/2}\lambda_z$	h	8.25	5.07	9.68	14.17	11.84
C_{max}	µg/mL	24.95	30.55	64.2	14.13	18.64
t_{max}	h	6	6	6	6	10
$AUC_{tissue}/AUC_{plasma}$	-	11.87	13.56	37.35	8.97	17.89

4.3 Third study. *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*)

4.3.1 Minimal inhibitory and minimal bactericidal concentration

All 10 isolates of *P. multocida* and all six isolates (including reference strain) of *E. coli* were susceptible to levofloxacin. None of the isolates were considered resistant. MIC and MBC values and MBC/MIC ratios in both media of all bacterial isolates are represented in Tables 4.5 and 4.6. Year of isolate collection is provided in Table 4.5, as well as diagnosis and origin of isolate.

Table 4.5

Minimal inhibitory concentration and minimal bactericidal concentration of *Pasteurella multocida* isolates from rabbits

	MIC_{broth} (µg/mL)	**MIC_{serum} (µg/mL)**	**MBC_{broth} (µg/mL)**	**MBC_{serum} (µg/mL)**	**MBC/MIC_{broth}**	**MBC/MIC_{serum}**	**Diagnosis and isolate origin**
P. multocida 297 (2021)	0.03	0.03	0.06	0.125	2	4	Nasal catarrh, pneumonia Nasal swab
P. multocida 320 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 306 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 122 (2021)	0.008	0.008	0.008	0.015	1	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 2101 (2021)	0.008	0.008	0.015	0.015	2	2	Rhinitis, Nasal swab
P. multocida 298 (2021)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 7697[a] (2022)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasal swab
P. multocida 3178 (2022)	0.008	0.008	0.125	0.125	16	16	Rhinitis, Nasolacrimal flush fluid
P. multocida 7042 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid
P. multocida 0634 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid

MIC – minimal inhibitory concentration, MBC minimal bactericidal concentration, a – *P. multocida* isolate selected for *in vitro* and *ex vivo* bacterial time-killing study

Table 4.6

Minimal inhibitory concentration and minimal bactericidal concentration of *Escherichia coli* reference strain ATCC25922 and isolates from rabbits

	MIC_{broth} (µg/mL)	MIC_{serum} (µg/mL)	MBC_{broth} (µg/mL)	MBC_{serum} (µg/mL)	MBC/MIC_{broth}	MBC/MIC_{serum}
E. coli ATCC 25922	0.03	-	0.03	-	1	-
E. coli 1[a]	0.03	0.03	0.25	0.25	8	8
E. coli 2	0.008	0.008	0.03	0.03	4	4
E. coli 5	0.015	0.015	0.06	0.06	4	4
E. coli 11	0.015	0.015	0.03	0.06	2	4
E. coli 12	0.008	0.008	0.03	0.03	4	4

MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.
[a]E. coli isolate selected for *in vitro* and *ex vivo* bacterial time-killing study

4.3.2 *In vitro* antibacterial activity of levofloxacin and time-killing curves

Figure 4.5 represents the time-dependent antibacterial activity of levofloxacin *in vitro* against a selected isolate of *P. multocida* (isolate No. 7697, MIC = 0.015 µg/mL).

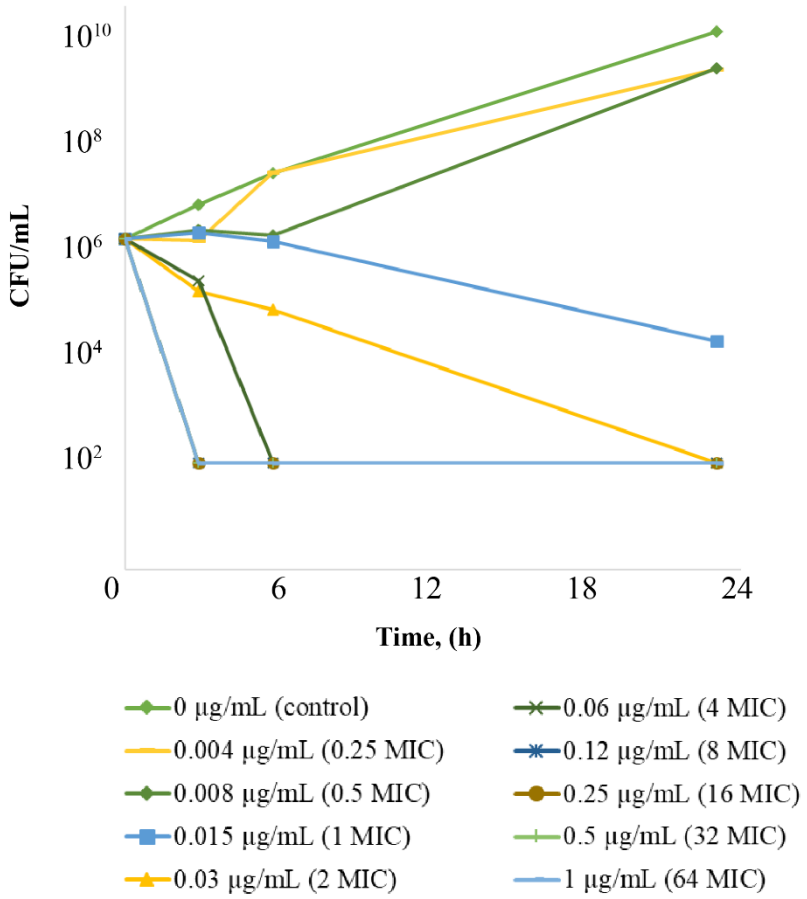

Figure 4.5 ***In vitro* time-killing curves representing the growth of *P. multocida* (No. 7697, MICw=0.015 µg/mL) with different levofloxacin concentrations in rabbit serum**

In the absence of the drug, the 24-hour incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.25 and 0.5 MIC were not able to inhibit bacterial growth, and after 24 hours of incubation, bacterial counts exceeded the initial inoculum. One MIC concentration reduced the bacterial growth, but after 24 hours of incubation, the bacterial count was similar to the initial inoculum. Concentrations of levofloxacin equal to 2 and 4 MIC reduced the number of bacteria gradually at 3 and 6 hours of incubation and eradicated the bacteria at 24 hours of incubation. Levofloxacin concentrations higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 hours of incubation.

Figure 4.6 shows the time-dependent antibacterial activity of levofloxacin *in vitro* against a selected isolate of *E. coli* (isolate No. 1, MIC = 0.03 µg/mL).

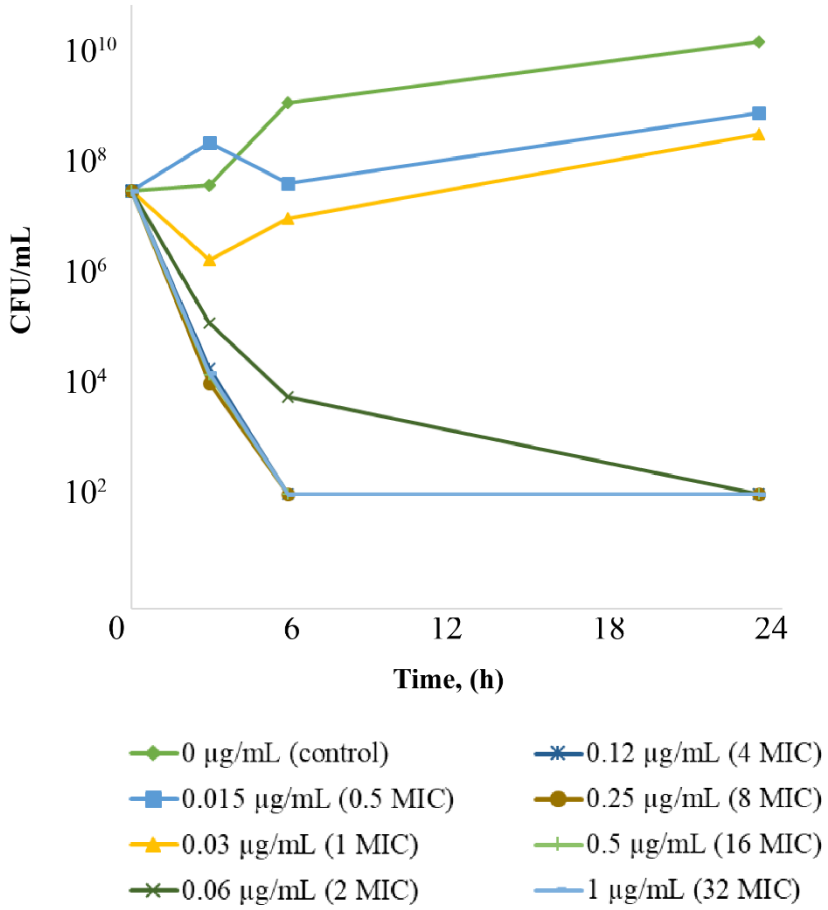

Figure 4.6 ***In vitro* time-killing curves representing the growth *E. coli* (No. 1, MIC=0.03 µg/mL) with different levofloxacin concentrations in rabbit serum**

In the absence of the drug, the 24-hour incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.5 and 1 MIC were not able to inhibit bacterial growth, and after 24 hours of incubation, bacterial counts exceeded the initial inoculum. Concentrations of levofloxacin equal to 2 MIC reduced the number of

bacteria gradually at 3 and 6 hours of incubation and eliminated the bacteria after 24 hours of incubation. Levofloxacin concentrations equal to and higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 hours of incubation.

4.3.3 *Ex vivo* antibacterial activity of levofloxacin after intramuscular and subcutaneous administration and time-killing curves

Figures 4.7 and 4.8 represent the bacterial time-killing curves for levofloxacin *ex vivo* against a selected isolate of *P. multocida* (isolate No. 7697, MIC = 0.015 µg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits.

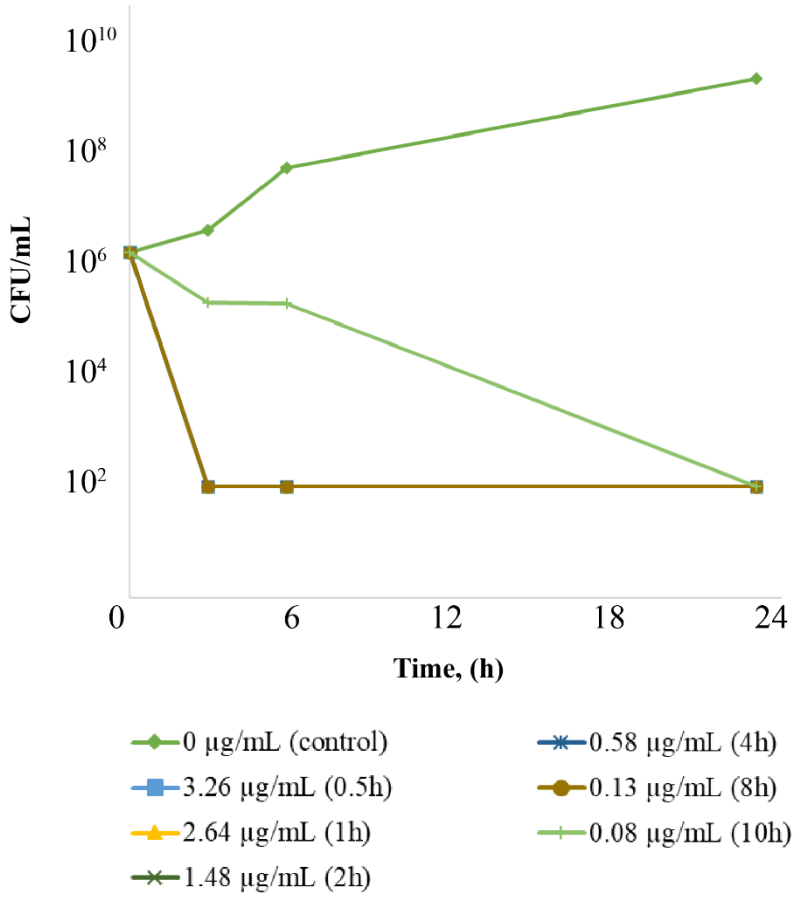

Figure 4.7 ***Ex vivo* time-killing curves representing the growth of *P. multocida* (No. 7697, MIC=0.015 µg/mL) with different levofloxacin concentrations in serum samples obtained after intramuscular administration of 5 mg/kg to healthy rabbits (n=6)**

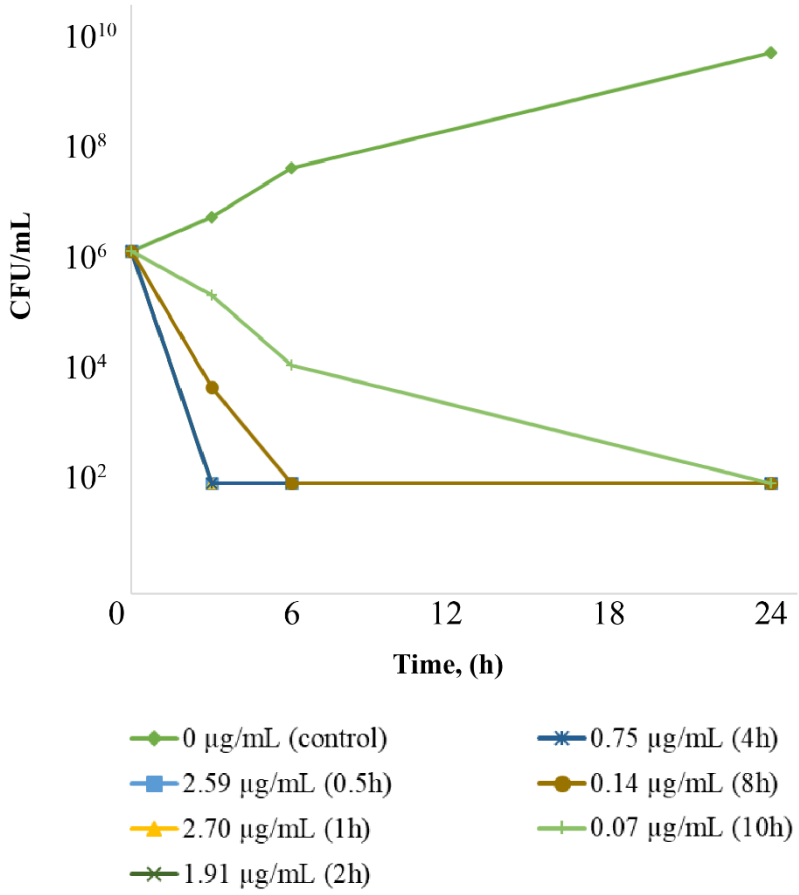

Figure 4.8 ***Ex vivo* time-killing curves representing the growth of *P. multocida* (No. 7697, MIC=0.015 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous administration of 5 mg/kg to healthy rabbits (n=4)**

Concentrations of levofloxacin achieved in serum after 0.5, 1, 2 and 4 hours of both IM and SC administration reduced the bacterial count to the limit of detection already after 3 hours of incubation. Considering 25 % protein binding, free levofloxacin concentrations in these serum samples were 2.45 (163 MIC), 1.98 (132 MIC), 1.11(74 MIC) and 0.44 (29 MIC) µg/mL, and 1.94 (130 MIC), 2.03 (135 MIC), 1.43 (96 MIC) and 0.56 (38 MIC) µg/mL for IM and SC samples, respectively. After incubation for 24 hours, all serum samples containing levofloxacin were able to reduce the *P. multocida* bacterial count to the limit of quantification.

Figures 4.9 and 4.10 represent the bacterial time-killing curves for levofloxacin *ex vivo* against a selected isolate of *E. coli* (isolate No. 1, MIC = 0.03 µg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits.

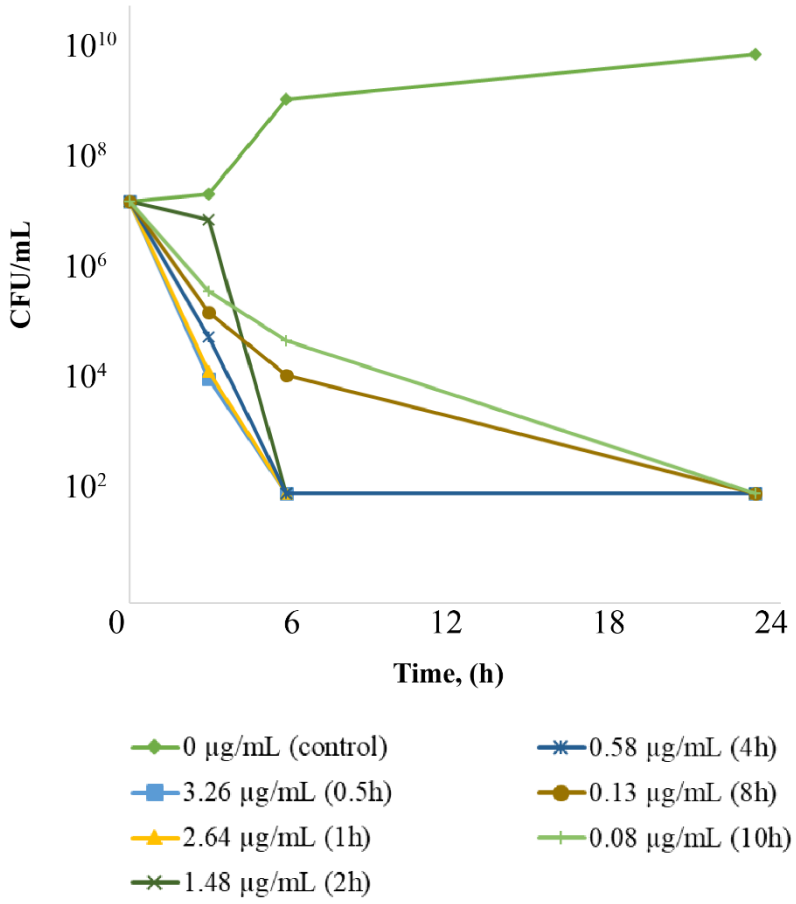

Figure 4.9 ***Ex vivo*** **time-killing curves representing the growth *E. coli* (No. 1, MIC=0.03 µg/mL) with different levofloxacin concentrations in samples obtained after intramuscular administration of 5 mg/kg to healthy rabbits (n=6)**

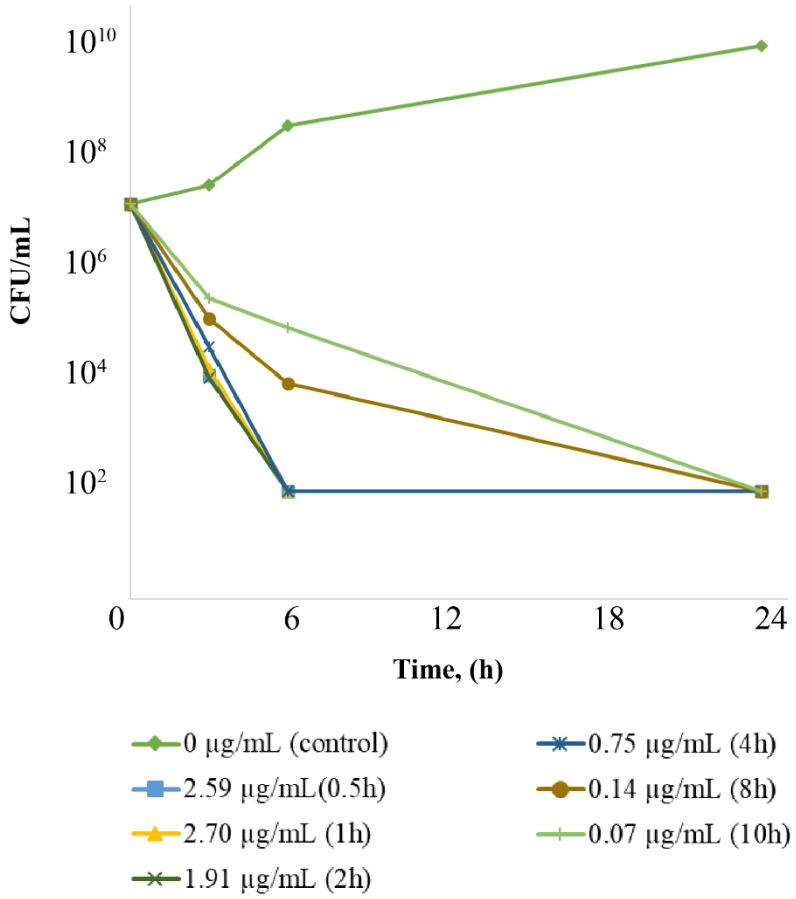

Figure 4.10 ***Ex vivo*** **time-killing curves representing the growth *E. coli* (No. 1, MIC=0.03 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous administration of 5 mg/kg to healthy rabbits (n=4)**

Only serum samples collected at 0.5, 1 and 2 hours, representing the highest drug concentrations, were able to reduce the bacterial count to the limit of quantification after 3 hours of incubation. Considering 25 % protein binding, free levofloxacin concentrations in these serum samples were 2.45 (82 MIC), 1.98 (66 MIC) and 1.11 (37 MIC) µg/mL, and 1.94 (65 MIC), 2.03 (68 MIC) and 1.43 (48 MIC) µg/mL for IM and SC samples, respectively. After incubation for 24 hours, all serum samples containing levofloxacin were able to reduce the *E. coli* bacterial count to the limit of quantification.

4.3.4 Pharmacodynamic modelling and daily dose calculation

For the pharmacodynamic analysis, the plots of AUC_{24}/MIC ratios versus changes in bacterial counts after 24 hours of incubation for selected *P. multocida* and *E. coli* isolates are presented in Figures 4.11 and 4.12, respectively. Pharmacodynamic data obtained from the I_{max} model, namely, AUC_{24}/MIC required for bacteriostatic, bactericidal and bacterial elimination for selected *P. multocida* and *E. coli* isolates, are presented in Tables 4.7 and 4.8, respectively.

Calculated daily doses of parenteral levofloxacin required to achieve antibacterial effects are reported in Table 4.9. Calculated daily doses for *P. multocida* isolates exhibiting highest MIC value (0.5 µg/mL) are 8.30, 11.55 and 30.18 mg/kg daily, for bacteriostatic, bactericidal and bacterial elimination effects, respectively.

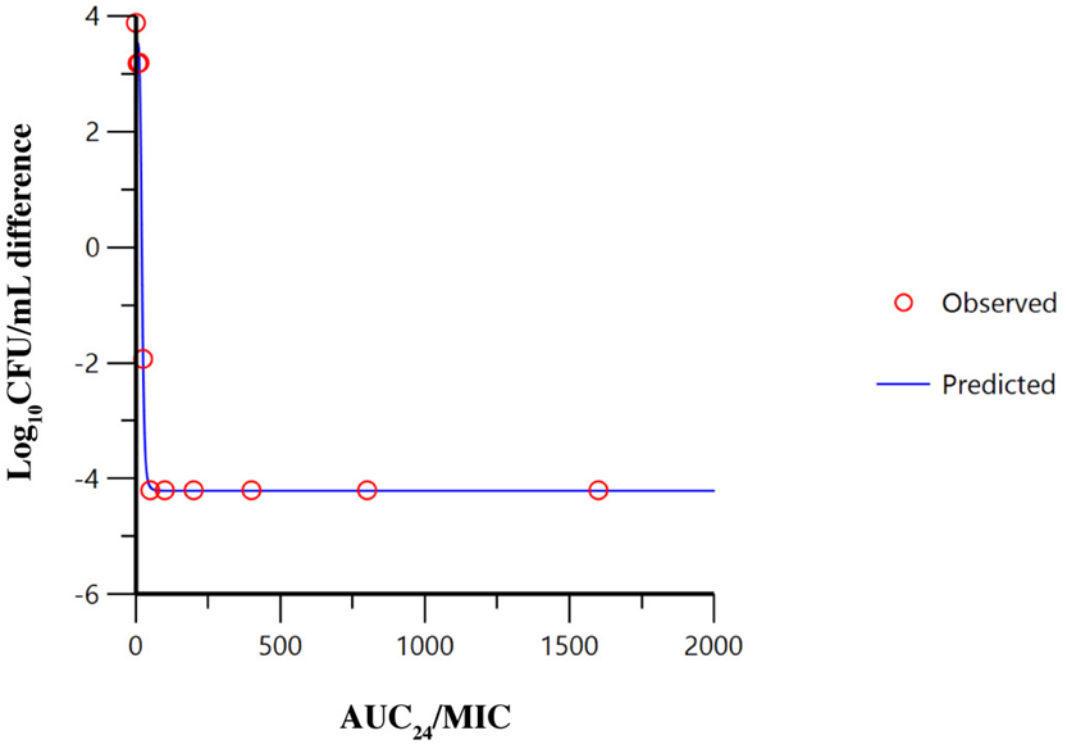

Figure 4.11 **Plot of *in vitro* AUC_{24}/MIC versus *P. multocida* (No. 7697, MIC=0.015 µg/mL) bacterial count difference in levofloxacin containing rabbit serum**

Table 4.7

Pharmacokinetic-pharmacodynamic levofloxacin data integration of *P. multocida* (No. 7697, MIC=0.015 µg/mL) *in vitro* growth inhibition

Parameter	Units	Estimated value
I_{max}	Log_{10} CFU/mL	7.75
E_0	Log_{10} CFU/mL	3.54
E_0- I_{max}	Log_{10} CFU/mL	–4.21
IC_{50}	h	21.41
AUC_{24}/MIC Bacteriostatic	h	20.76
AUC_{24}/MIC Bactericidal	h	28.88
AUC_{24}/MIC Bacterial elimination	h	75.46
Slope (γ)	N/A	5.64

I_{max} – difference between log_{10} difference in bacterial count between 0 and 24 h in the control sample ($logE_0$) and the log_{10} difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached E_0 – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in the control sample E_0 - I_{max} – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached IC_{50} – AUC_{24}/MIC producing 50 % of the maximal antibacterial effect γ – the Hill coefficient, slope of the AUC_{24}/MIC response curve N/A – not applicable.

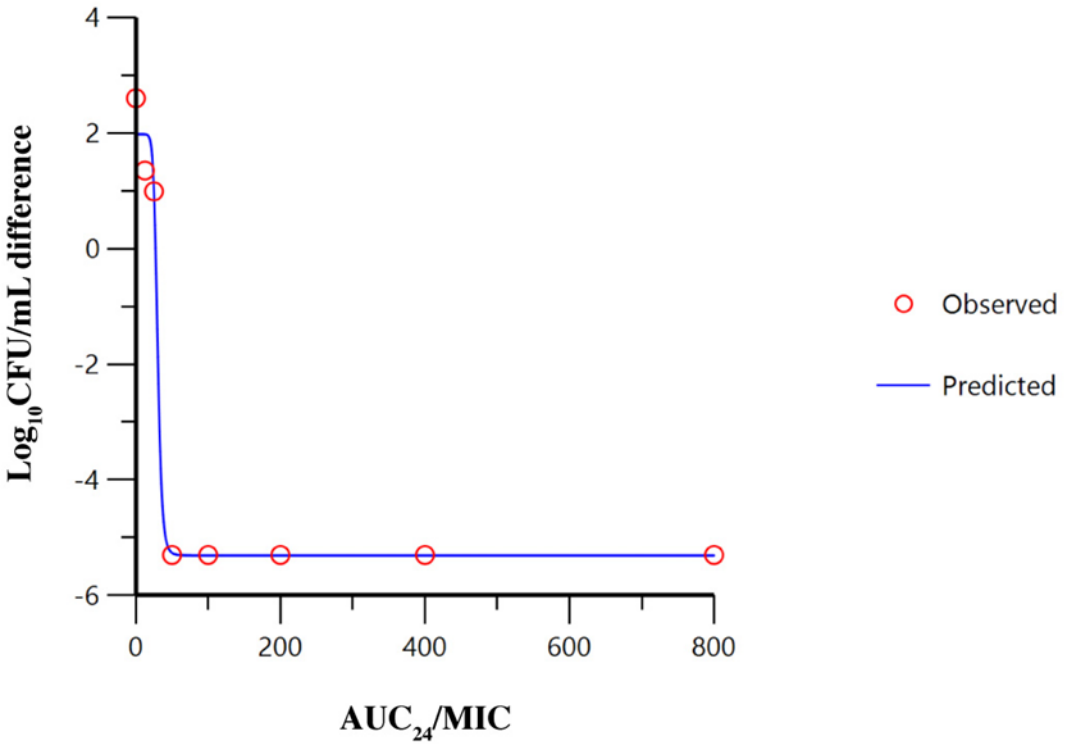

Figure 4.12 **Plot of *in vitro* AUC_{24}/MIC versus *E. coli* (No. 1, MIC=0.03 µg/mL) bacterial count difference in levofloxacin containing rabbit serum**

Table 4.8

Pharmacokinetic-pharmacodynamic levofloxacin data integration of *E. coli* (No. 1, MIC=0.03 µg/mL) *in vitro* growth inhibition

Parameter	Units	Estimated value
I_{max}	Log_{10} CFU/mL	7.28
E_0	Log_{10} CFU/mL	1.98
E_0- I_{max}	Log_{10} CFU/mL	–5.30
IC_{50}	h	30.08
AUC_{24}/MIC Bacteriostatic	h	27.25
AUC_{24}/MIC Bactericidal	h	32.49
AUC_{24}/MIC Bacterial elimination	h	59.62
Slope (γ)	N/A	9.98

I_{max} – difference between log_{10} difference in bacterial count between 0 and 24 hours in the control sample ($logE_0$) and the log_{10} difference in bacterial count in the sample incubated with levofloxacin for 24 hours when the limit of detection of 100 CFU/mL is reached E_0 – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in the control sample E_0 - I_{max} – log_{10} difference in the bacterial count from 0 to 24 hours of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached IC_{50} – AUC_{24}/MIC producing 50 % of the maximal antibacterial effect γ – the Hill coefficient, slope of the AUC_{24}/MIC response curve N/A – not applicable

Table 4.9

Calculated daily doses of levofloxacin for parenteral administration to rabbits against *P. multocida* (MIC=0.015 µg/mL) and *E. coli* (MIC=0.03 µg/mL)

Dose per day	*P. multocida* (MIC=0.015 µg/mL)	*E. coli* (MIC=0.03 µg/mL)
Bacteriostatic effect	0.25 mg/kg	0.65 mg/kg
Bactericidal effect	0.35 mg/kg	0.78 mg/kg
Bacterial elimination	0.91 mg/kg	1.43 mg/kg

5 Discussion

5.1 First study. Pharmacokinetics of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits

To the best of our knowledge, this is the first-time levofloxacin PK profiles after IM and SC administration in healthy rabbits were evaluated, although IV administration had been examined previously in rabbits infected by *S. pneumoniae*.

The 5 mg/kg dose used in this study was based on the dose used previously in a levofloxacin study involving broiler chicken (Lee et al., 2017). This dose is within the range of doses previously used in other mammalian and bird species (Aboubakr, 2012; Aboubakr & Soliman, 2014; Albarellos et al., 2005; Kumar et al., 2012; Urzúa et al., 2020; Varia et al., 2009); a dose associated with reduced risks of side effects. One rabbit died during the current experiment, and the death may be attributed to the stress of the sampling procedures. While necropsy showed no noticeable organ changes in the rabbit, a single IV dose of levofloxacin in humans has been reported to produce cardiovascular side effects – increased heart rate and QT interval prolongation (Basyigit et al., 2005). Thus, cardiovascular effects may also be involved in the lethal outcome in this individual.

All 3 routes of administration (IV, IM, and SC) used in this study produced very similar results for key pharmacokinetic parameters. This could be explained by the fast absorption and rapid distribution of the drug after the extravascular administration routes mimicking the pharmacokinetic profile of the IV administration. In this study, the AUC values for all 3 routes of administration were similar, and there was complete (calculated over 100 %) systemic bioavailability of levofloxacin reported following both IM and SC administration. Maximal plasma concentrations for both extravascular routes were reached at around the same time (30–45 min post-administration) and were of similar value (around 3 μg/mL). Similar parallel results were observed for SC and IM mean residence times, clearances, and volumes of distribution compared to those for IV administration. These similarities in PKs suggest that the same drug efficacy should be expected for all 3 routes of administration when levofloxacin is given at a dose of 5 mg/kg. Moreover, previous studies of other fluoroquinolones in rabbits (Fernandez-Varon et al., 2005; Marín et al., 2008) and of levofloxacin in other animal species (Lee et al., 2017; Madsen et al., 2019; Patel et al., 2012) showed very similar pharmacokinetic profiles after different routes of administration. The levofloxacin terminal plasma half-life appeared to be one of the shortest among the species tested (1.8–2.06 hours, depending on the route of parenteral administration).

The volume of drug distribution at a steady-state after IV administration of 1.37 mL/g suggests moderate penetration of the drug through the biological membranes of the body. This

value is within the range reported in avian and mammalian species, 0.56 mL/g in sheep (Sartini et al., 2020) and 2.88–3.25 mL/g in broiler chickens (Lee et al., 2017; Varia et al., 2009).

The results of the non-compartmental PK analysis showed that bioavailability values after IM and subcutaneous SC administration exceeded 100 %. Complete bioavailability of levofloxacin after extravascular administration has also been reported in other species (Vercelli et al., 2020, Goudah & Abo-El-Sooud, 2009; Lee et al., 2017; Madsen et al., 2019, Sartini et al., 2020). Interestingly, other fluoroquinolones studied in rabbits after IM and SC administration have also shown complete bioavailability, with actual values exceeding 100 % (Fernandez-Varon et al., 2007; Marín et al., 2008; Marín et al., 2018). This may be due to various factors that have already described in the literature (Brown, 1996; Martinez et al., 2006; Toutain & Bousquet-Mélou, 2004a), e.g. non-linear clearance. The IM administration of orbifloxacin, norfloxacin, danofloxacin, and marbofloxacin have all been reported to exceed the 100 % bioavailability level in rabbits (Abo-El-Sooud & Goudah, 2010; Fernandez-Varon et al., 2005; Marín et al., 2008; Marín et al., 2018). Moreover, SC ofloxacin, orbifloxacin, and danofloxacin administration to rabbits also showed complete bioavailability (Fernandez-Varon et al., 2007; Marangos et al., 1997; Marín et al., 2008). These observations indicate that, in general, fluoroquinolones are well absorbed and widely distributed after IM or SC administration in rabbits. The application of compartmental PK analysis using PKanalix software (Lixoft, Simulations Plus, USA) to the same levofloxacin rabbit plasma concentrations supported the complete levofloxacin bioavailability in rabbits after the parenteral administration. IM administration data was best fitted to the two-compartmental with central and peripheral compartments and a linear elimination model with first order absorption. Akaike's Information Criterion was applied to determine the goodness of fit. The mean bioavailability was calculated to be 97 %. SC administration data was best fitted to the one compartment and a linear elimination model with first order absorption. The mean bioavailability was calculated to be 108 %.

Compared to the study in rabbits infected with *S. pneumoniae* (Destache et al., 2001), the AUC values of levofloxacin were much lower (at least twice corrected to the dose administered) in the present study. The plasma terminal half-lives of the drug were at least 3 times longer than that observed in our study. These differences might be due to differences in rabbit breed (New Zealand white vs. cross-bred in this study), size of the animals in the 2 studies (2–3 kg vs. 4.2 kg in the study performed in the scope of this Thesis) and the provision of other drugs (e.g. anaesthetic administration in). Additionally, the presence of infection may have slowed the elimination of the drug from the body in a manner similar to that observed in a PK study of marbofloxacin in infected rabbits (Abo-El-Sooud & Goudah, 2010).

The AUC values reported for rabbits appear to be the lowest among the other species studied, taking into account the administered dose differences. This might be related to the rapid elimination of the drug from the rabbit body. The average plasma clearance of levofloxacin was 0.6 mL/g×h with some variability among the study animals. This is the highest clearance rate thus far reported in all previous mammalian and avian species studied, except sheep, which had similar reported clearance (0.55 mL/g×h (Patel et al., 2012) vs. 0.6 mL/g×h in rabbits) and half-life of elimination (2.38 hours vs. 2.06 hours in rabbits) values. However, another study in sheep showed a lower clearance of 0.2 mL/g×h and a longer elimination half-life (3.3 hours), but that study was performed using sheep with a body mass almost twice as large, possibly, resulting in slower drug elimination (Goudah & Hasabelnaby, 2010). The longest levofloxacin elimination half-life after the extravascular administration is currently reported in Asian elephants (up to 12.11 hours) by Kilburn et al. (2022). The high rate of elimination in rabbits may be due to their high cardiac output and heart rate (Mitchell & Tully, 2008). Higher clearance in rabbits is observed after administration of other fluoroquinolones; orbifloxacin, norfloxacin, danofloxacin, and moxifloxacin are cleared even faster than levofloxacin with clearance values of 0.9, 0.8, 0.8, and 0.8 mL×g/h, respectively (Fernandez-Varon et al., 2005; Fernandez-Varon et al., 2007; Marín et al., 2008; Marín et al., 2018). These results indicate that parenteral fluoroquinolone administration in rabbits will require frequent dosing. Alternatively, the route of administration could be changed to consider practitioners' convenience and/or reduction of the handling stress of the infected animal.

A low extraction ratio (around 7 %) may indicate that levofloxacin is not fully metabolised and may be excreted unchanged by the kidney (Brown, 1996; Martinez et al., 2006). This suggests the use of orally administered dosage forms (Toutain & Bousquet-Mélou, 2004b). Although extraction ratio values were not computed in other species in which levofloxacin pharmacokinetics were established, we calculated approximate extraction ratios for the above-mentioned studies. Low levofloxacin extraction ratios were predicted in cats, dogs, and rabbits (around 2 %) based on the clearance and mean animal body weights (Albarellos et al., 2005; Destache et al., 2001; Landoni & Albarellos, 2019; Madsen et al., 2019). In food-producing animals, the levofloxacin extraction rate is also low. Based on data provided in the literature for goats (Goudah & Abo-El-Sooud, 2009), sheep (Goudah & Hasabelnaby, 2010; Patel et al., 2012), and camels (Goudah, 2009) the values are 3.2 %, 3.9 %, and 9.5 %, respectively. The estimated extraction ratio values in all of the animal species investigated indicate similar drug elimination abilities among the species.

As the elimination half-life of levofloxacin for all 3 routes of administration was short, frequent administration, which is potentially stressful to the animal, would be required. The

authors, therefore, do not suggest than any of these parenteral routes are suitable for regular clinical use of levofloxacin in the studied dosage form. While the therapeutic efficacy of fluoroquinolones may be inferred through pharmacokinetic-pharmacodynamic surrogate index assessment and the use of the AUC_{24}/MIC ratio, the low AUC value and the inability to quantify levofloxacin in rabbit plasma at 24 hours post drug administration resulted in the inability to perform these surrogate calculations based on the experimental data. Based on the results of this study, a dose of 5 mg/kg of levofloxacin is unlikely to produce a therapeutic effect in rabbits. The calculated effective daily dose for levofloxacin, based on an *Enterobacteriaceae* MIC value of 0.5 μg/mL reported in dogs (Madsen et al., 2019), was 29 ± 8 mg/kg, The estimate is in agreement with the oral dose of 25 mg/kg in dogs supposed to attain similar therapeutic targets. In rabbit management, the oral route for drug administration (in medicated feed or water) is the most common one used. Levofloxacin is reported to have complete oral bioavailability in 2 pet mammalian species; dog (104 ± 30 %) (Albarellos et al., 2005; Madsen et al., 2019) and cat (86 ± 43 %) (Albarellos et al., 2005). If this trend in oral bioavailability is similar in rabbits, the effective daily dose of levofloxacin reported in our study could be added to pelleted rabbit food or drinking water. However, as infected animals may lose their appetite while maintaining water intake, we suggest the daily dose could be prepared in 50–100 mL of drinking water (i.e. the average daily water intake of rabbits) (Harcourt-Brown, 2002).

This study is the first to investigate the effect of systemic administration of levofloxacin on some ocular parameters. The high variability in the qualitative parameters of tears between individual animals before and after treatment with levofloxacin made identification of trends difficult. The authors suggest that the dose may have been too small or a single administration insufficient to produce any discernible effects on tear production. The basal level of the tear production assessed with STT method (7 ± 3 mm/min) was slightly higher than those reported for English angora rabbits and Dutch rabbits (5.4 and 4.6 mm/min, respectively) (Rajaei et al., 2016). Regardless, tear osmolarity appeared to decrease slightly but significantly ($p = 0.002$) at 48 hours after drug administration. Therefore, we suggest that levofloxacin administration at 5 mg/kg is unlikely to cause major changes in the qualitative and quantitative properties of tears. However, studies with multiple-dose administration and a larger number of animals are warranted to make solid conclusions.

According to obtained study results, a levofloxacin dose of 5 mg/kg is unlikely to be effective in rabbits. Moreover, a single administration of that dose is unlikely to have any effect on tear parameters. Based on the calculations, a daily dose of 29 mg/kg may be effective for IV administration of levofloxacin.

5.2 Second study. Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in geese

The geese did not show any adverse effects during or after drug treatments. The dose was chosen on the basis of a previous study on chickens (Lee et al., 2017). This is the first study which dealt with the pharmacokinetics of levofloxacin in geese. The drug showed a moderate half-life (7.39 hours) comparable with results from chickens (6.93 hours, (Lee et al., 2017), but was longer than in ducks (2.76 hours), with a slower clearance (geese, 0.28 mL/g×h; ducks, 0.41 mL/g×h) (Aboubakr & Soliman, 2014). The V_{ss} in geese (1.40 mL/g) was in line with the value found in ducks (1.37 mL/g). Levofloxacin showed higher AUC (7.59 μg×h/mL), if normalised for dose, than values reported in ducks (4.89 μg×h/mL) and chicken (5.09 μg×h/mL) (Aboubakr & Soliman, 2014; Lee et al., 2017). Species specific differences, such as variations in metabolic pathways, plasma protein binding or differences in absorption processes, may have caused these variances. After oral administration, levofloxacin showed faster (t_{max}) and higher (C_{max}) absorption in geese than ducks, turkeys and chickens (Aboubakr & Soliman, 2014; Aboubakr et al., 2014; Lee et al., 2017; Patel et al., 2012; Varia et al., 2009). The different formulations administered, variability in experimental design, climatic conditions or feed management might have contributed to such differences. Levofloxacin's oral bioavailability is high in avian species in general (ducks, 73.6 %; chickens, 59.5 %; leghorn hens, 71.6 %; turkeys, 79.9 %), but is highest in geese (95.6 %), suggesting that the oral route is an appropriate route of administration in birds, and especially geese (Aboubakr & Soliman, 2014; Aboubakr et al., 2014; Patel et al., 2012; Varia et al., 2009).

The MIC of levofloxacin has not yet been determined for bacteria isolated from geese. Regarding the AUC_{24} value obtained in the present study after oral administration (5 mg/kg), levofloxacin in geese appeared be effective against bacteria at an MIC <0.24 μg/mL. For the MIC against *E. coli* isolated in broilers (0.125 μg/m, Lee et al., 2017), an AUC_{24}/MIC ratio of 136 was obtained, which suggests that the dose regimen in the present study might be effective in geese. Levofloxacin's plasma protein binding has not been evaluated in geese, but has resulted in a low percentage (25 %) in broilers (Lee et al., 2017) and may be considered negligible for the pharmacokinetic-pharmacodynamic surrogate calculation. However, further studies are required to establish if the plasma protein binding of levofloxacin in geese is in line with that found in other avian species.

Levofloxacin was detected in all tissues selected, and the concentration was highest at 6 hours and gradually decreased over 48 hours. Considering that in humans approximately 90 % of levofloxacin is rapidly absorbed from the intestinal tract into the hepatic portal vein and, similarly to other fluoroquinolones, is primarily excreted unchanged from the kidney in the

urine (Fish & Chow, 1997). Hence, it was reasonable to expect a higher drug residue in liver and kidney in geese. Probable tropisms related to levofloxacin have not yet been evaluated. The tissue depletion profile found in the present study was in line with that found in chickens (Kyuchukova et al., 2013; Lee et al., 2017). In this study, muscle levofloxacin concentrations, normalised for dose, were higher than concentrations found in chickens (Kyuchukova et al., 2013; Lee et al., 2017). These differences could be due to species specific difference, or the diverse analytical techniques used.

The MRL for fluoroquinolones in poultry liver is about 0.1 μg/g (EMA, 1997, 1999, 2002). On the basis of this value, a preliminary withdrawal time has been computed with the CI of 95 % for liver, resulting in a time of 89.7 hours. Despite the fact that this matched well with the data reported in chickens – 4 days (Ravikumar et al., 2015), caution should be taken because of the small population sample size. Further studies are required to confirm this finding. Drug penetration in tissue can be described using the $AUC_{tissue}/AUC_{plasma}$ ratio. A ratio value over 1 indicates relatively higher drug concentrations in the tissue than in blood, with potential for tissue accumulation (Bellmann et al., 2004). The $AUC_{tissue}/AUC_{plasma}$ ratios in our study were high in all tissues, and especially in liver. Further studies could clarify this point (e.g. whether levofloxacin may be stored specifically in hepatocytes).

5.3 Third study. *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*)

To the best of our knowledge, this study describes for the first-time levofloxacin time-killing curves for *P. multocida* and *E. coli* isolates from rabbits. None of the bacterial isolates included in this study showed resistance to levofloxacin. However, reports are indicating cases of *P. multocida* and *E. coli* resistance to this drug (Saha et al., 2021; Sitovs et al., 2021). MIC values for both *P. multocida* and *E. coli* were low, compared to other pathogens' MIC reported in the literature (See Annex 5, Table A2). Two *P. multocida* isolates (No. 7042 and 0634) showed relatively high MIC (0.5 μg/mL). As no clinical breakpoints for levofloxacin for *P. multocida* isolates from rabbits currently exist, applying CLSI M100 (CLSI, 2018b) levofloxacin breakpoints, these isolates could be considered susceptible. Applying fluoroquinolone clinical breakpoints for respiratory *P. multocida* (pradofloxacin, enrofloxacin and danofloxacin) according to the CLSI VET08 (CLSI, 2018a), these isolates would not be considered susceptible, anymore (susceptible defined as MIC ≤ 0.25 μg/mL), but rather intermediate. All other *P. multocida* isolates showed MIC values (0.008–0.03 μg/mL) in line with MIC_{90} values reported for veterinary fluoroquinolones and their active metabolites – difloxacin, enrofloxacin, ciprofloxacin, marbofloxacin, orbifloxacin and pradofloxacin

(0.008–0.05 μg/mL) against *P. multocida* (Riviere & Papich, 2018). MIC_{90} values for the same veterinary fluoroquinolones against *E. coli* (0.03–0.39 μg/mL) were slightly higher compared to *E. coli* MIC values obtained in the present study (0.008–0.03 μg/ mL). Only 15 bacterial isolates were used in our study; thus, it is not yet obvious that levofloxacin is significantly superior to other veterinary fluoroquinolones.

Minimal bactericidal concentration/MIC ratios of levofloxacin were not high in the present study. The median ratios for *P. multocida* and *E. coli* isolates were 2 and 4, respectively. That is similar to ratios obtained from isolates from humans in which, levofloxacin was reported to achieve a reduction in CFU/ mL of ≥ 99.9 % of most aetiology of bacteremia faster compared to other fluoroquinolones (Akinjogunla et al., 2022). MBC/MIC ratios >8 were reported to be associated with antibiotic tolerance (Gonzalez et al., 2013). Our pharmacodynamic study results do not suggest levofloxacin tolerance in rabbits.

AUC_{24}/MIC is described as the most important factor to determine efficacy of concentration-dependent antibacterial drugs, including fluoroquinolones (Aliabadi & Lees, 2001). In the present study, the use of *ex vivo* AUC_{24}/MIC was not suitable for pharmacokinetic–pharmacodynamic modelling. The reason for that was bacterial count reduction to the detection limit after 24 hours of incubation with all experimentally obtained levofloxacin concentrations in rabbit serum. All samples from time points collected after IM and SC dose of 5 mg/kg had levofloxacin concentrations higher than 1 MIC for both bacterial isolates used in the time-killing study. *In vitro* AUC_{24}/MIC data were used for modelling instead. AUC_{24}/MIC values obtained for lower levofloxacin concentrations (0.25, 0.5 and 1 MIC, which did not reduce the bacterial counts to the detection limit) provided more data for creating the model. When time-killing curves for *in vitro* and *ex vivo* experiments were visually compared, their similarity provided almost identical bacterial killing patterns. That justifies the use of *in vitro* AUC_{24}/MIC data for modelling.

Slightly slower killing rate was observed in the *in vitro* study compared to the *ex vivo* study. That could be attributable to chemical differences between experimental rabbit serum and commercially available rabbit serum used for the *in vitro* study. Hill coefficient values in both models in this study were high, 5.64 for *P. multocida* and 9.98 for *E. coli*, respectively. These values illustrate the rapid increase in levofloxacin activity with the small increase in the concentration. A slightly less steep slope of 5.21 for levofloxacin against *E. coli* isolated from broiler chickens is reported (Lee et al., 2017). Levofloxacin in this study showed similar AUC_{24}/MIC ratios required for bacteriostatic, bactericidal and bacterial elimination effects for *P. multocida* (20.76, 28.88 and 75.46 hours), compared to marbofloxacin, (20.9, 45.2 and 71.7 hours) for *P. multocida* isolates from pigs (Dorey et al., 2017) and slightly lower than

marbofloxacin for isolates from calves (48.6, 64.9 and 74.8 hours, respectively) (Potter et al., 2013). AUC_{24}/MIC ratios for bacteriostatic, bactericidal and bacterial elimination effects in this study for *E. coli* (27.25, 32.49 and 59.62 hours) were higher compared to values reported in chickens –18.77, 24.02 and 36.27 hours, respectively (Lee et al., 2017). AUC_{24}/MIC ratios obtained by for danofloxacin against *E. coli* isolated from turkeys were significantly lower (0.42, 1.90 and 6.73 hours) (Haritova et al., 2006) and for enrofloxacin against *E. coli* isolated from chickens were much higher (257.40 and 2794.40 hours for bacteriostatic effect and bacterial elimination, respectively) (Haritova & Russenova, 2010). Despite the previous conclusion from our levofloxacin pharmacokinetic study in rabbits, that a dose of 5 mg/kg levofloxacin is unlikely to be effective in rabbits, the *ex vivo* time-killing curves showed a reduction of the bacterial counts to the limit of quantification at 24 hours. Calculated daily doses appear to be even lower. In our pharmacokinetic-pharmacodynamic study, proposed doses per day required for bacteriostatic, bactericidal and bacterial elimination effects (0.25–1.43 mg/kg daily) were lower compared to the levofloxacin doses calculated for broilers (1.1–4.3 mg/kg daily) (Lee et al., 2017) and for rabbits – 29 mg/kg daily (Sitovs et al., 2020). Previously reported dose was up to 100-fold higher that doses obtained in this study. Compared to the dose reported by previously, this study utilises experimental pharmacodynamic data from susceptible bacterial time-killing curves, while previous pharmacodynamic data were from the published literature. Difference in doses between two studies originates from the higher AUC_{24}/MIC used in calculations – 72 hours, as reported the literature (Madsen et al., 2019) and with lower MIC values used in calculations. In the current study, doses were calculated based on the experimentally obtained MIC values, while previously we used MIC = 0.5 μg/mL (Sitovs et al., 2020). Doses calculated using highest *P. multocida* MIC (0.5 μg/mL) are less different from the dose reported in the rabbit levofloxacin pharmacokinetics study, 8.30, 11.55 and 30.18 vs. 29 mg/kg daily. Real, rather than theoretical MIC values were used in dose calculations here. As we determined that levofloxacin bioavailability in rabbits after IM and SC routes of administration is around 100 % it is considered complete. From the point of view of bioavailability, there is no difference between IM and SC administration for suggested daily doses. However, compared to SC, the IM administration is generally more painful and considering relatively small muscle mass in rabbits, rarely used (Shellim, 2011). Additional factors that can contribute to the calculation of daily doses are associated with changes in fluoroquinolone pharmacokinetics in rabbits in the diseased state. For example, *P. multocida* infection results in the change in the primary pharmacokinetic parameter clearance for marbofloxacin (Abo-El-Sooud & Goudah, 2010). If the same could apply to levofloxacin, that may impact the calculation of the dose. To prove this, an additional pharmacokinetic study of

levofloxacin in infected animals would be required. There are also some known limitations in our study. First, a small number of animals in the pharmacokinetic study do not cover all possible inter-animal difference in clearance, necessary for dose calculation. Impact of infection was not considered in this study, as serum samples from healthy rabbits were used. Small number of bacterial isolates used in this study does not represent all MIC variability within one isolate and among population of wild-type pathogenic bacteria in rabbits. The *ex vivo* study does not take into account the immune response of the animal organism, which could contribute to the elimination of bacteria and possibly allow lower doses of the antimicrobial agent to be used. The effect of inoculum concentration was not assessed in terms of antimicrobial activity of levofloxacin. Finally, this study did not predict further resistance development against levofloxacin for the tested microbial isolates, and no mutant prevention concentrations values were obtained in this study. However, fluoroquinolone resistance is an important issue in global health (Brown, 1996; WHO, 2019). Lastly, consideration of antimicrobial stewardship principles (Lloyd & Page, 2018) in the selection and possible use of levofloxacin in rabbits has to be considered.

Conclusions

Levofloxacin shows favourable pharmacokinetic profiles and is generally well tolerated in rabbits and geese. Levofloxacin dose of 5 mg/kg is likely to be effective in studied animal species and even lower doses are active for highly susceptible bacteria. Our studies provide preliminary examination of key elements of the dose regimen in rabbits and geese. Highest concentrations of levofloxacin were observed in the liver and kidneys, suggesting possible drug accumulation.

The results of this study do not encourage the use of levofloxacin instead of conventional veterinary antibiotics, but provide and up-to-date information on levofloxacin, that will help veterinary practitioners and scientists to make informed choices regarding appropriate levofloxacin use.

Proposals

Safe and effective use of an antibiotic requires require additional issues to be addressed.

1. Despite susceptibility of microbial isolates have been reported in multiple studies, it does not exclude further resistance development. The resistance development mechanisms and resistance possibility against levofloxacin for the microbial isolates of interest is advised to be evaluated using the mutant-prevention concentration determination.
2. The dose optimisation for levofloxacin in veterinary medicine is advised to be performed. This could be achieved by using population pharmacokinetics methods and utilizing extensive MIC data from microorganisms of interest.
3. The impact of the infected state is advised to be evaluated in order to account for the pharmacokinetic differences in real clinical cases where levofloxacin could be used.
4. Levofloxacin MRL values for food producing are advised to be defined in countries where levofloxacin is used in food-producing animals.

Publications and reports on topics of Thesis

Publications

1. **Sitovs, A.**, Voiko, L., Kustovs, D., Kovalcuka, L., Bandere, D., Purvina, S., & Giorgi, M. (2020). Pharmacokinetic profiles of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits (*Oryctolagus cuniculus*). Journal of veterinary science, 21(2), e32. https://doi.org/10.4142/jvs.2020.21.e32
2. Sartini, I., Łebkowska-Wieruszewska, B., **Sitovs, A.**, Lisowski, A., Poapolathep, A., & Giorgi, M. (2021). Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese. British poultry science, 62(2), 193–198. https://doi.org/10.1080/00071668.2020.1842855
3. **Sitovs, A.**, Sartini, I., & Giorgi, M. (2021). Levofloxacin in veterinary medicine: a literature review. Research in veterinary science, 137, 111–126. https://doi.org/10.1016/j.rvsc.2021.04.031
4. **Sitovs, A.**, Skadins, I., Purvina, S., & Bandere, D. (2023). *In vitro* and *ex vivo* antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*) – A preliminary study. Journal of veterinary pharmacology and therapeutics, 10.1111/jvp.13383. Advance online publication. https://doi.org/10.1111/jvp.13383

Reports and theses at international congresses and conferences

1. **Sitovs A.**, Kustovs D., Giorgi M., Kovalcuka L., Voiko L., Purviņa S., Bandere D. (2019). Levofloxacin Assay in Rabbit Plasma: UPLC Method Optimisation and Validation – Rīga Stradiņš University International Conference on Medical and Health Care Sciences "Knowledge for Use in Practice": Abstracts April 1–3, 2019, 396.
2. **Sitovs A.**, Kustovs D., Giorgi M., Kovalcuka L., Voiko L., Purviņa S., Bandere D. (2019). Pharmacokinetics of levofloxacin after three different routes of single parenteral administration to domestic rabbits – 4th conference "Current approach to health and diseases in animals and humans" Lublin, Poland: Abstracts, September 19–20, 2019, Oral presentation II.O.7
3. **Sitovs, A.**, Voiko L., Purviņa S., Bandere D., Giorgi M. (2020). Levofloxacin pharmacokinetics and efficacy prediction in domestic rabbits (*Oryctolagus cuniculus*). ICECVM 2nd International Conference of the European College of Veterinary Microbiology, Online: Abstracts, October 25–27, 2020, Oral presentation
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Levofloxacin in veterinary medicine: a literature review

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ABSTRACT

A potent third-generation antimicrobial fluoroquinolone drug, levofloxacin was introduced into human clinical practice in 1993. Levofloxacin is also used in veterinary medicine, however its use is limited: it is completely banned for veterinary use in the EU, and used extralabel in only companion animals in the USA. Since its introduction to clinical practice, many studies have been published on levofloxacin in animal species, including pharmacokinetic studies, tissue drug depletion, efficacy, and animal microbial isolate susceptibility to levofloxacin. This literature overview highlights the most clinically relevant and scientifically important levofloxacin studies linked to the field of veterinary medicine.

1. Introduction

Although more commonly used in human medicine, the fluoroquinolone antimicrobial agent levofloxacin has also been a focus of research in veterinary medicine. Levofloxacin is a third-generation fluoroquinolone drug. Compared to previous generations of fluoroquinolones, it possesses expanded activity against Gram-positive bacteria and atypical intracellular pathogens (North et al., 1998). Indications for levofloxacin in human medicine include chronic bronchitis, acute sinusitis, inhalational anthrax (post-exposure), nosocomial and community-acquired pneumonia, prostatitis, pyelonephritis, skin and soft tissue infections and urinary tract infections. Levofloxacin is a drug included in the World Health Organization's List of Essential Medicines (WHO, 2019).

Several research papers reporting on levofloxacin in non-human animals have been published in recent years (Casas et al., 2019; Vercelli et al., 2020; Sartini et al., 2020a, 2020b; Wang et al., 2021), indicating an increasing interest in levofloxacin as an extralabel drug in companion and food-producing animals. This interest is likley due to many of the currently approved antimicrobial agents for veterinary use not meeting the needs of veterinarians in the management of antibiotic-resistant infections (Papich, 2020). With emerging resistance to fluoroquinolones of first and second generations, worldwide (WHO, 2012), levofloxacin is used in animals in many countries, including both registered and extralabel uses. In non-EU countries (e.g., Argentina, India, China, and Russia) levofloxacin is registered as a veterinary drug (Table 1), whereas in the USA, all veterinary use is extralabel. In the USA, the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA, 1994) allows the use of extralabel drugs registered for use in other species or humans, when the health of an animal is threatened, or when suffering or death may result from failure to treat. This has led to inexpensive generic human levofloxacin tablets being used in pet animals (Papich, 2020) and the inclusion of levofloxacin in the International Society for Companion Animal Infectious Diseases Guidelines for the Diagnosis and Management of Bacterial Urinary Tract Infections in Dogs and Cats (Weese et al., 2019). However, it is of importance to note that under the AMDUCA, extralabel use of fluoroquinolones, including levofloxacin, is prohibited in food animals. The use of levofloxacin in verteinary medicine in the countries described above is in stark contrast to the countries of the EU, where all non-veterinary fluoroquinolones are deemed "critically important in human medicine and their use in animals should be restricted to mitigate the risk to public health" by the European Medical Agency (i. e. they are included in the "restricted" category B), and so are not used in non-human animals (EMA, 2020). The differing restictions on the use of levofloxacin in veterinary medicine worldwide has lead to the availability of a large volume of information on its use, however such information has not been compiled into a single source. This review intends to summarize the existing data from the veterinary field related to levofloxacin, so veterinary care professionals worldwide can evaluate the appropriateness of its use in their practice. As such, human medicine studies are mostly avoided in this review. Detailed information about levofloxacin use in humans can be

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found in the review of North et al. (1998).

The Scopus database (keywords: "levofloxacin" and "veterinary") and references of the research papers found were used as data sources. In cases where full-text articles were unable to be sourced, data cited is from the abstracts only. Additionally, although some research data was sourced from domestic journals, only peer-reviewed publications were considered. This resulted in 43 research articles on levofloxacin pharmacokinetics in mammals and birds, 8 tissue depletion articles and 111 articles referring to the antimicrobial activity (*in vivo* and *in vitro*) of levofloxacin against microorganisms isolated from various animal species and/or their primary habitats.

2. Description and physicochemical properties

Levofloxacin (chemical name: (S)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (Fig. 1); molecular mass 361.37 g/mol; pharmaceutically available as a heminydrate, ($C_{18}H_{20}FN_3O_4 \cdot {}^1/_2H_2O$; 370.38 g/mol)) is the optical S-(-) isomer of ofloxacin. Ofloxacin is a racemic mixture, but most of its antimicrobial activity is due to the S-isomer, which is 32- to 128-fold more potent than the R-isomer. Levofloxacin was developed to take advantage of this antimicrobial potency, which requires approximately half the usual dose of ofloxacin to achieve

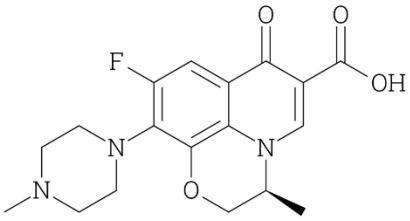

Fig. 1. Levofloxacin chemical structure.

Table 1
Veterinary formulations containing levofloxacin.

Country	Name	Active ingredient(s)	Dosage form	Manufacturer	Species	Indication	Dosing	Treatment duration	Withdrawal time	Reference
Argentina	Floxaday	Levofloxacin	Tablets	Holliday - Scott S. A.	Dog	Soft tissue/ respiratory/ prostate/ mammary gland infections, UTI, osteomyelitis, septicemia, pyoderma	PO 10 mg/kg every 24h	N/A	N/A	https://www.floxaday.com.ar/
Argentina	Floxaday	Levofloxacin	Injectable solution	Holliday - Scott S. A.	Dog		1.5 mL /10 kg every 24h	N/A	N/A	
China	ZDHF-Levofloxacin W.S. P	Levofloxacin	Powder	Shijiazhuang ZDHF Stock-Raising Co., Ltd	Fowl	Increase poultry laying rate	100g of the powder + 150kg water, twice daily	3-5 days	N/A	http://www.cnleader.com.cn/
India	LEVOVET	Levofloxacin	Powder	Paramount Agrovet (P) Ltd	N/A	N/A	N/A	N/A	N/A	http://vetneedsgroup.com/products/poultry-medicines/levovet/
India	Veterinary Levofloxacin Injection	Levofloxacin	Injectable solution	Zuche Pharmaceuticals Private Ltd	N/A	N/A	N/A	N/A	N/A	http://facmedpharma.com/products/veterinary-medicine-manufacturer/injection
India	Levosept	Levofloxacin, colistin	Oral liquid	Vetbiochem India Private Ltd	Poultry	N/A	N/A	N/A	N/A	https://www.vetbiochem.in/search.html?ss=Levosept
India	LCB Vet	Levofloxacin, colistin and bromhexine	Oral liquid	Vetline (A Divison of Simfa Labs Pvt. Ltd)	N/A	N/A	4·8 mL per 10 L of drinking water	3·5 days	Meat: 28 days; Eggs: 7days	http://vetline.in/lcb-vet.html
Russia	Лексофлон (Leksoflon)	Levofloxacin	Injectable solution	NITA-FARM	Cattle, pig	N/A	IM injection 1 mL per 30 kg BW	3-5 days	Cattle/Pig (meat): 9 days; Milk: 4 days	https://www.nita-farm.ru/produktsiya/leksoflon/
Russia	Лексофлон OR (Leksoflon OR)	Levofloxacin	Oral liquid	NITA-FARM	Poultry, pig	N/A	1 mL per 20 kg BW (0.5 mL per 1 L drinking water)	3-5 days	Poultry (meat): 7 days; Pigs (meat): 9 days	https://www.nita-farm.ru/produktsiya/leksoflon-or/

BW – body weight, N/A – data not available in the reference source.

similar efficacy, with a reduced toxicity profile.

Levofloxacin is a light-sensitive, pale yellow-white to yellow-white crystal or crystalline powder, and is odorless with a bitter taste. It expresses slightly acidic (carboxylic acid moiety dissociation constant of 6.24 (Nowara et al., 1997)) and strongly lipophilic properties (log Kow = -0.39, logP = 2.1). It is soluble in dimethyl sulfoxide, dimethyl formamide, glacial acetic acid and chloroform, slightly soluble in ethanol, sparingly soluble in water, and practically insoluble in ether. At a pH range of 0.6-5.8, levofloxacin water solubility is essentially constant at approximately 100 pg/mL (sparingly soluble). Above pH 5.8, the solubility increases rapidly to a maximum at pH 6.7 (272 pg/mL) (North et al., 1998; https://infectweb.com/product/cravit/).

3. History

Levofloxacin was patented in 1985 by Daiichi Seiyaku Pharmaceutical Co. Ltd in Japan, but was not introduced to the human pharmaceutical market until 1993, when it was produced as oral tablets under the brand name Cravit®. Also in 1993, Daiichi Sankyo entered into a licensing agreement with Sanofi-Aventis, and levofloxacin was subsequently marketed and sold under the trade name Tavanic®. Since 2010, generic formulations have also been available. Levofloxacin is currently registered for human use by both the United States Food and Drug Administration (FDA) and EMA, with a variety of formulations available. Oral tablets, oral, injectable and ophthalmic solutions are approved for use in human medicine in the USA (FDA, 2021). Oral tablets, injectable and ophthalmic solutions are approved in the EU (EMA, 2019).

4. Pharmacology

4.1. Mechanism of action

Like other fluoroquinolones, levofloxacin inhibits bacterial DNA gyrase (an enzyme required for DNA replication, transcription, repair, and recombination) and topoisomerase IV, thereby inhibiting the introduction of single-strand breaks on bacterial chromosomes, and resealing them after supercoiling. This prevents bacterial DNA replication and transcription, leading to a bactericidal effect.

4.2. Use of levofloxacin

Levofloxacin is effective in the treatment of a variety of infectious diseases. Its spectrum of activity includes Gram-positive aerobic bacteria, Gram-negative aerobic bacteria, some anaerobic bacteria, and other microorganisms including *Chlamydia* spp., *Mycoplasma* spp., and *Mycobacterium* spp. Similar to human levofloxacin, veterinary levofloxacin is available as both oral and parenteral forms in non-EU countries (Table 1). These products are used for farm animals with infectious disease (Al Masud et al., 2020); Лексофлон (Leksoflon), for example, is indicated for the treatment of infections caused by the microorganisms listed in Table 2.

Table 2
Reported antimicrobial spectrum of activity of veterinary levofloxacin formulation Лексофлон (Leksoflon)

Gram-positive	Gram-negative	Atypical intracellular
Clostridium spp.	Bacteroides spp.	Chlamydia spp.
Listeria monocytogenes	Campylobacter spp.	Mycoplasma spp.
Staphylococcus spp.	Enterobacter spp.	Rickettsia spp.
Streptococcus spp.	E. coli	
	Fusobacterium spp.	
	Haemophilus spp.	
	Moraxella spp.	
	Pasteurella spp.	
	Proteus spp.	
	Pseudomonas aeruginosa	
	Salmonella spp.	

To achieve maximum therapeutic efficacy, adequate susceptibility of the microorganism to the therapeutic agent is required. Often, pharmacokinetic-pharmacodynamic (PK/PD) surrogate indices such as area under the concentration *vs* time curve divided by the minimal inhibitory concentration (AUC/MIC) are applied (Toutain et al., 2002) to predict therapeutic efficacy. The AUC/MIC target for fluoroquinolones derived from human data was originally estimated at over 125 hours (McKellar et al., 2004), however, more recent studies have suggested a lower value of 72 hours (Madsen et al., 2019). Susceptibility and MIC values for levofloxacin have been reported for multiple microorganisms isolated from animal sources, however as no veterinary-specific breakpoint values are available for levofloxacin, human medical breakpoints have been used. It is of great importance that, according to the Clinical and Laboratory Standards Institute VET09 report (CLSI, 2019a), susceptibility test results interpretations based on human breakpoints should be made with low confidence in the correlation between *in vitro* results and clinical outcomes in animals. Authors of most of the publications included in this review used susceptibility breakpoint values from the Performance Standards for Antimicrobial Susceptibility Testing (28th edition, supplement M100; CLSI, 2018). According to these standards, levofloxacin MIC breakpoints for most of the microorganisms are as follows: susceptible = ≤2 μg/mL, intermediate = 4 μg/mL, resistant = ≥8 μg/mL; and for the disk diffusion method, zone diameter breakpoints: suceptible = zone diameter ≥17 mm, intermediate = 14-16 mm, resistant = ≤13 mm. It is of importance to note that the newer CLSI rationale document (CLSI, 2019b) suggests different breakpoint values for *Enterobacteriaceae* and *Pseudomonas aureginosa*: *Enterobacteriaceae* susceptible = ≤0.5 μg/mL, intermediate = 1 μg/mL, resistant = ≥2 μg/mL; *P. aureginosa* susceptible = ≤1 μg/mL, intermediate = 2 μg/mL, resistant = ≥4 μg/mL.

4.3. Microbial resistance

Microbial resistance to fluoroquinolones may result from mutations in defined regions of DNA gyrase or topoisomerase IV (i.e., quinolone resistance determining regions (QRDRs) – gyrA and parC) or altered efflux. The development of microbial resistance to levofloxacin has been studied in human medicine, however there is limited research in other animal species. Mutations in microbial genes isolated from animals associated with increased resistance to levofloxacin, such as an increase in efflux pump expression, have been doucumented in molecular studies in a variety of microorganisms, including *Escherichia coli* (Liu et al., 2012; Cheng et al., 2020), *Riemerella anatipestifer* (Sun et al., 2012), *Salmonella* spp. (Kang and Woo, 2014; Kim et al., 2013), *Haemophilus parasuis* (Zhao et al., 2018), and *Staphylococcus aureus* (Suzuki et al., 2016). Of interest, a pharmacokinetic/pharmacodynamic study by Vercelli et al. (2020) also identified an increase in levofloxacin resistance of *E. coli* isolated from goats within 48 hours of low dose (2 mg/kg bodyweight) parenteral levofloxacin administration, however the authors did not investigate the underlying mechanism for this finding.

4.4. Antimicrobial activity of levofloxacin

4.4.1. Gram-negative microorganisms

Gram-negative bacterial susceptibility to levofloxacin are presented in Tables 3a, 3b and 3c, with data expressed as reported (i.e., if only the percentage of resistant strains were reported, the percentage of sensitive strains was not calculated herein). As a result, some data is missing or incomplete (e.g., sampling period, MIC value or type of tissues sampled); the same approach has been taken for all other tables in this review. More than 30 studies evaluated the susceptibility of *E. coli* isolated from animals to levofloxacin (Tables 3a and 3b), some of which report almost complete resistance (Anes et al., 2020; Benameur et al., 2019). Additionally, an increase in the percentage of resistant *E. coli* strains from

Table 3a
Susceptibility of Gram-negative microorganisms isolated from animals to levofloxacin

Bacteria	Animal species	Health status	Country	Sampling period	N	Sample type	S %	I %	R %	MIC	Reference
Various (19 species; mostly *P. mirabilis*)	Owl monkey (*Aotus azarai infulatus*)	Healthy	Brazil	2011	N/A	Swabs	100.0			0.12	Da Silva et al., 2013
Haemophilus parasuis (BP)	Pig	Diseased	China	2008-2010	73	Tissue			24.7	N/A	Zhang et al., 2014
Haemophilus parasuis (NBP)	Pig	Diseased	China	2008-2010	37	Tissue			24.3	N/A	Zhang et al., 2014
Haemophilus parasuis	Pig	Diseased	China	2014-2017	143	Tissue			20.3	<0.25–128	Zhao et al., 2018
Haemophilus parasuis	Pig	Diseased	China	2007-2008	110	N/A	93.6			0.008–16	Zhou et al., 2010
Brucella abortus	Cattle	Diseased	Mexico	2012	3	Feces	100.0			N/A	Morales-Estrada et al., 2016
Brucella melitensis	Cattle	Diseased	Mexico	2012	3	Feces	66.0		33.0	N/A	Morales-Estrada et al., 2016
Brucella suis	Cattle	Diseased	Mexico	2012	1	Feces	100.0			N/A	Morales-Estrada et al., 2016
Brucella abortus	Goat	Diseased	Mexico	2012	3	Feces	100.0			N/A	Morales-Estrada et al., 2016
Bordetella hinzii	Turkey	Diseased	USA	2004	1	Swabs, tissue	100.0			N/A	Beach et al., 2012
Bordetella avium	Turkey	Diseased	USA	Pre1979–2010	12	Swabs, tissue		8.33		N/A	Beach et al., 2012
Bordetella avium	Saw-whet owl Aegolius acadicus)	Healthy	USA	2006	1	Swabs, tissue	100.0			N/A	Beach et al., 2012
Francisella tularensis subsp. *holarctica*	Various (hare, vole)	N/A	Spain	N/A	32	N/A	S			<0.25	del Blanco et al., 2004
Aeromonas hydrophilia	Tilapia	Diseased	Malaysia	2019	1	Tissue	100.0			N/A	Pauzi et al., 2020
Aeromonas hydrophilia	Rainbow trout (*Oncorhynchus mykiss*)	N/A	Bulgaria	N/A	12	Swabs	100.0			N/A	Stratev et al., 2013
Klebsiella pneumoniae	Cattle	Diseased	India	N/A		Milk	S			N/A	Arya et al., 2020
Escherichia coli	Various (pig, chicken, duck)	Diseased	China	2002-2010	495	Feces, tissues			70.5	0.0625 - >256	Liu et al., 2012
Escherichia coli	Dog	Diseased	Japan	2009-2012	38	Swabs			18.4	N/A	Inoue et al., 2013
Escherichia coli (ESBL 34%)	Rat	N/A	Gabon	2010	32	Feces			56.3	N/A	Onanga et al., 2020
Escherichia coli	Pig	N/A	China	2014-2017	479	Wastewater			38.8	N/A	Cheng et al., 2020
Escherichia coli (ESBL)	Cattle	Diseased	India	N/A	30	Feces, milk			74.7	N/A	Prajapati et al., 2020
Escherichia coli (MDR)	Cattle	N/A	Ireland	2007	12	N/A			100.0	N/A	Anes et al., 2020
Escherichia coli (ESBL)	Cattle	Healthy	India	2018	22	Milk	83.3	16.7	0.0	N/A	Batabyal et al., 2018
Escherichia coli	Cattle	Diseased	India	N/A	31	Feces	87.1			N/A	Boyal et al., 2018
Escherichia coli	Cattle	Healthy	Bangladesh	N/A	2	Milk	S			N/A	Tanzin et al., 2016
Escherichia coli	Buffalo	Healthy	Bangladesh	N/A	1	Milk	S			N/A	Tanzin et al., 2016

BP – biofilm producing, NBP – non-biofilm producing, ESBL – extended-spectrum beta-lactamases, MDR – multidrug resistant, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source.

1993 to 2013 was reported by Chen et al. (2014). Many studies investigating the efficacy of levofloxacin in other Gram-negative infections have been undertaken in mouse models (Judy et al., 2009; Safi et al., 2013; Safi et al., 2014; Isogai et al., 2001; Klimpel et al., 2008). Judy et al. (2009) reported that levofloxacin resulted in 100% animal survival, but failed to fully eradicate *Burkholderia mallei* (MIC 2.5 μg/mL), whereas Safi et al.'s (2013, 2014), studies indicated that levofloxacin alone and in combination with rifampicin is effective in *Brucella melitensis* infections. Isogai et al. (2001) found it effective to use levofloxacin together with anti-TNF-α antibodies against enterohaemorrhagic *E. coli*, and Klimpel et al. (2008) demonstrated levofloxacin efficacy against a seemingly lethal dose (intra-nasal infection with approximately 99 colony-forming units) of *Francisella tularensis* and subsequent antibody development post-treatment.

4.4.2. Gram-positive and other microorganisms

The susceptability of Gram-positive and atypical microorganisms to levofloxacin are presented in Tables 4a and 4b. The majority of investigated microbes of this class have susceptibility to levofloxacin, e.g., a retrospective study of dog osteomyelitis showed that less than 10% of

Table 3b
Susceptibility of Gram-negative microorganisms isolated from animals to levofloxacin

Bacteria	Animal species	Health status	Country	Sampling period	N	Sample type	S %	I %	R %	MIC	Reference
Escherichia coli	Buffalo	Diseased	India	N/A	15	Swabs	66.6			N/A	Bhadaniya et al., 2019
Escherichia coli	Cattle	Diseased	India	2009-2011	30	Milk	96.7	3.3	0.0	N/A	Mohanty et al., 2013
Escherichia coli (MDR)	Cattle	Healthy	Nigeria	2006-2008	500	Feces	4.6			N/A	Ajayi et al., 2011
Escherichia coli (31 STEC and 6 EPEC)	Yak	N/A	India	N/A	37	Milk, milk products	100.0			N/A	Bandyopadhyay et al., 2012
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	1993-1995	91	Feces, tissues			13.2	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	1996-2000	95	Feces, tissues			22.1	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	2001-2005	112	Feces, tissues			40.2	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	2006-2010	112	Feces, tissues			53.6	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	2011-2013	130	Feces, tissues			54.6	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	China	1993-2013	540	Feces, tissues			38.7	N/A	Chen et al., 2014
Escherichia coli	Pig	Healthy, diseased	China	2003-2005	203	Feces, tissues			50.2	N/A	Jiang et al., 2011
Escherichia coli	Poultry (Chicken, geese, duck, partridge)	Healthy, diseased	China	2003-2005	389	Feces, tissues			20.8	N/A	Jiang et al., 2011
Escherichia coli	Pig, poultry	Healthy	China	2003-2005	300	Feces, tissues			14.0	N/A	Jiang et al., 2011
Escherichia coli	Pig, poultry	Diseased	China	2003-2005	292	Feces, tissues			48.3	N/A	Jiang et al., 2011
Escherichia coli	Duck	Healthy	China	2003-2005	10	Feces			0.0	N/A	Jiang et al., 2011
Escherichia coli	Chicken (Broiler breeder)	Healthy	Algeria	2017-2018	37	Tissues			89.1	N/A	Benameur et al., 2019
Escherichia coli	Chicken	Diseased	Egypt	2015-2016	34	Tissues, yolk sac	38.2	35.3	26.5	N/A	Ibrahim et al., 2019
Escherichia coli (90% APEC)	Chicken (Broiler)	Diseased (suspected)	Nepal	2016-2017	50	Tissues			50.0	N/A	Subedi et al., 2018
Escherichia coli	Chicken (Broiler)	Healthy	Bangladesh	N/A	54	Swabs, feces			22.0	N/A	Mahmud et al., 2018
Escherichia coli (APEC)	Chicken (Broiler)	Diseased	USA	1996-2000	56	Tissues	98.0		2.0	0.25 - 8	Zhao et al., 2005
Escherichia coli	Duck	Diseased	India	N/A	25	Tissues	S			N/A	Panda et al., 2010
Escherichia coli	Pigeon	Healthy	Bangladesh	2017	21	Swabs, feces	100.0		0.0	N/A	Karim et al., 2020
Enterobacter hormaechei (ESBL)	Green turtle (*Chelonia mydas*)	Diseased	Brazil	2016	1	Tissues	100.0			N/A	Goldberg et al., 2019
Citrobacter freundii	Green turtle (*Chelonia mydas*)	Diseased	Brazil	2016	1	Tissues	100.0			N/A	Goldberg et al., 2019
Vibrio vulnificus	Seal (*Phoca largha*)	Diseased	China	2016	1	Tissues	S			N/A	Li et al., 2018
Vibrio spp.	Horse mackerel (*Trachurus trachurus*)	N/A	Turkey	2006	9	Tissues	100.0			N/A	Özer et al., 2008
Pseudomonas spp.	Buffalo	Diseased	India	N/A	3	Swabs	66.6			N/A	Bhadaniya et al., 2019
Pseudomonas aeruginosa	Dog	Healthy	South Korea	2017-2018	38	Swabs			13.2	0.015 - 32	Park et al., 2020

ESBL – extended-spectrum beta-lactamases, MDR – multidrug resistant, STEC – Shiga toxin producing *E. coli*, EPEC – Enteropathogenic *E.coli*, APEC – avian pathogenic *E. coli*, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source.

various isolated microorganisms were resistant to this drug (Siqueira et al., 2014). However, there are some exceptions. Multiple studies (Rodriguez-Palacios et al., 2006; Álvarez-Pérez et al., 2013; Álvarez-Pérez et al., 2014b) have indicated complete clostridial resistance to levofloxacin, and Sasaki et al. (2007) and Ruscher et al. (2010) reported complete *Staphylococcus pseudintermedius* resistance to levofloxacin in dogs. Studies into the suceptability of *S. aureus* have reported mixed results: Fernandez et al. (1999) reported that oral administration of levofloxacin was more effective than ciprofloxacin in rabbits with *S. aureus* abscesses, whereas ophthalmic administration in rabbits was not effective in the reduction of keratitis caused by a resistant *S. aureus* strain (Tungsiripat et al., 2003). Similarly, an *in vitro* pharmacokinetic model of bulbar conjunctiva of rabbits reported a stronger bactericidal effect of 1.5% levofloxacin ophthalmic solution compared to 0.5% solution against different MIC *S. aureus* strains (Suzuki et al., 2016). Interestingly, Backues and Wiedner (2019) identified levofloxacin as the fluoroquniolone of choice in elephant tuberculosis, despite an earlier study by Miller et al. (2018) reporting unsuccessful treatment of *Mycobacterium tuberculosis* infection in captive elephants due to poor complience and adverse effects.

Rabbits infected with *Bacillus anthracis* (MIC 0.12 μg/mL) showed high survival rates, suggesting that intravenous levofloxacin is an effective therapeutic agent against inhalational anthrax (Yee et al., 2010). Oral administration of levofloxacin was also effective in the anthrax model in Rhesus monkeys, where an initial dose of 15 mg/kg followed by 4 mg/kg every 12 hours prevented morbidity and mortality

Table 3c
Susceptibility of Gram-negative microorganisms isolated from animals to levofloxacin

Bacteria	Animal species	Health status	Country	Sampling period	N	Sample type	S %	I %	R %	MIC	Reference
Pseudomonas aeruginosa	Dog	Diseased	South Korea		46	Swabs			15.2	0.015 – 32	Park et al., 2020
Pseudomonas aeruginosa	Dog	Diseased	USA	2003-2006	106	Swabs			16.0	0.015 – 32	Rubin et al., 2008
Pseudomonas aeruginosa	Dog	Diseased	USA	3 years	27	Swabs	100.0	0.0	0.0	N/A	Ledbetter et al., 2007
Pseudomonas aeruginosa	Mink	Dead	China	2007-2015	69	Tissues, soil			13.0	16 - 128 (R)	Bai et al., 2019
Pseudomonas aeruginosa	Mink	Diseased/ dead	China	2010-2011	30	Feces, feed, tissues			13.3	N/A	Qi et al., 2014
Pseudomonas aeruginosa	Chicken	N/A	Egypt	2018	33	Environment swabs, yolk sac	100.0	0.0	0.0	N/A	Eraky et al., 2020
Pseudomonas aeruginosa	Chicken	Diseased/ dead	Egypt	N/A	42	Tissues, yolk sac	73.8	7.2	19.0	N/A	Farghaly et al., 2017
Proteus mirabilis	Various (Dog, cat)	Diseased	Portugal	1999-2015	107	N/A		0.0	7.5	N/A	Marques et al., 2019
Proteus mirabilis (BP)	Various (Dog, mink, cattle, fowl)	Diseased	China	2014-2016	162	Feces	57.4	18.5	24.1	N/A	Sun et al., 2020
Proteus mirabilis (NBP)	Various (Dog, mink, cattle, fowl)	Diseased	China	2014-2016	14	Feces	57.1	0.0	42.9	N/A	Sun et al., 2020
Proteus mirabilis	Turtle	N/A	South Korea	N/A	15	Feces	73.0	20.0	7.0	0.03 - 8	Pathirana et al., 2018
Proteus vulgaris	Turtle	N/A	South Korea	N/A	7	Feces	85.7	14.3	0.0	0.03 - 4	Pathirana et al., 2018
Proteus hauseri	Turtle	N/A	South Korea	N/A	2	Feces	100.0			0.06	Pathirana et al., 2018
Proteus vulgaris	Human (Catfish wound)	Diseased	USA	N/A	1	Swabs	100.0			<0.25	Huang et al., 2013
Helicobacter suis	Various (Pig, monkey)	N/A	Belgium	N/A	35	Tissues			5.7	0.03 - 32	Berlamont et al., 2019
Shigella sonnei	Yak	Diseased	China	2014-2016	44	Feces			9.1	N/A	Zhu et al., 2018
Salmonella typhimurium	Guinea pig	N/A	Peru	2016, 2018	35	N/A	60.0	14.3	24.7	N/A	Huamán et al., 2020
Salmonella spp.	Poultry	N/A	India	N/A	30	Feces, eggs		3.3	93.3	N/A	Tamuly et al., 2008
Salmonella spp.	Chicken (Broiler)	Diseased	Egypt	2017-2019	5	Tissues	60.0	40.0		N/A	Badr et al., 2020
Salmonella spp.	Chicken	N/A	Egypt	N/A	19	Tissues	78.9		15.8	N/A	Elfeil et al., 2020
Salmonella spp.	Duck	Diseased, dead	Bangladesh	N/A	19	Tissues, feces	S			N/A	Rahman et al., 2016
Salmonella spp.	Pigeon	Diseased, dead	Bangladesh	N/A	12	Tissues, feces	S			N/A	Rahman et al., 2016
Salmonella spp.	Pigeon	Healthy	Bangladesh	N/A	11	Swabs, feces			18.2	N/A	Karim et al., 2020
Acinetobacter spp.	Cattle	Healthy	South Korea	N/A	176	Milk	100.0		0	N/A	Gurung et al., 2013
Acinetobacter baumannii	Cattle	Healthy	South Korea	N/A	57	Milk	100.0		0	N/A	Gurung et al., 2013
Acinetobacter baumannii	Chicken	Healthy	Iraq	2017-2019	80	Tissues			37.5	N/A	Kanaan et al., 2020
Acinetobacter baumannii	Turkey	Healthy	Iraq	2017-2019	120	Tissues			37.5	N/A	Kanaan et al., 2020
Fusobacterium spp.	Buffalo	Diseased	India	N/A	5	Swabs	100.0			N/A	Bhadaniya et al., 2019

BP – biofilm producing, NBP – non-biofilm producing, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source.

and did not cause development of microbial resistance (Kao et al., 2006). Finally, a topical formulation containing levofloxacin, miconazole, and dexamethasone was found to be effective in external otitis management in cats (Barbieri Bastos et al., 2019), and in buffalos intrauterine co-administration of levofloxacin with ornidazole and α-tocopherol was effective in treating and preventing postpartum affection (Markandeya et al., 2011).

4.5. Adverse effects

Levofloxacin side effects have been comprehensivley documented in human medicine, and encompass common gastrointestinal effects (nausea, diarrhea, constipation), headache, insomnia, dizziness, and rare, but severe tendinitis and peripheral neuropathy (Liu, 2010). However, reports of side effects in animals are limited. Most of the studies included in this review performed a single dose administration (dose range 2 – 810 mg/kg body weight) and not all of them reported on side effects. Of those that did report on side effects, most suggested a lack of side effects associated with levofloxacin treatment (Casas et al., 2019; Urzúa et al., 2020; Landoni and Albarellos, 2019; Albarellos et al., 2005; Dumka and Srivastava, 2006; Vercelli et al., 2020; Goudah and Abo-El-Sooud, 2009; Sartini et al., 2020a; Patel et al., 2012a; Goudah and Hasabelnaby, 2010; Goudah et al., 2008; Goudah, 2008; Bisht et al., 2018; Lee et al., 2017; Patel et al., 2012b; Varia et al., 2009; Aboubakr, 2012; Aboubakr, 2012; Sartini et al., 2020b; Aboubakr and Soliman, 2014), however Madsen et al. (2019) reported transient vomiting, soft

Table 4a
Susceptibility of Gram-positive microorganisms isolated from animals against levofloxacin

Bacteria	Animal species	Health status	Country	Sampling period	N	Sample type	S %	I %	R %	MIC	Reference
Various	Cattle	Healthy, diseased	India	N/A	31	Lavage	87.1			N/A	Bajaj et al., 2018
Staphylococcus spp.	Cattle	Diseased	Croatia	N/A	53	Milk			<5%	N/A	Zdolec et al., 2016
Staphylococcus spp.	Cattle	Healthy	Croatia	N/A	41	Milk			<5%	N/A	Zdolec et al., 2016
Staphylococcus spp.	Cattle	Diseased	India	N/A	68	Milk	88.2	8.8	2.9	N/A	Mohanty et al., 2013
Staphylococcus spp.	Buffalo	Diseased	India	N/A	15	Swabs	66.6			N/A	Bhadaniya et al., 2019
Staphylococcus pseudintermedius (MRSP)	Various (dog, cat, horse, donkey)	Diseased	Germany	2005-2008	146	N/A	2.1	0.0	97.9	<1 - 4	Ruscher et al., 2010
Staphylococcus pseudintermedius	Dog	Healthy, diseased	South Korea	N/A	49	Swabs			34.7	N/A	Kang and Woo, 2014
Staphylococcus pseudintermedius (MRSP)	Dog	Healthy, diseased	Japan	2006	18	Swabs			100.0	8 - >8	Sasaki et al., 2007
Staphylococcus intermedius	Dog	Healthy, diseased	Italy	2006-2007	114	Swabs	98.2			N/A	Vanni et al., 2009
Staphylococcus schleiferi	Dog	Healthy, diseased	Italy	2006-2007	8	Swabs	37.5			N/A	Vanni et al., 2009
Staphylococcus aureus	Dog	Diseased	India	N/A	6	Swabs	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus	Pig	N/A	India	N/A	2	Swabs		50.0	50.0	N/A	Sharma et al., 2020
Staphylococcus aureus	Cattle	Diseased	India	N/A	28	Milk	82.1	10.7	7.1	N/A	Sharma et al., 2020
Staphylococcus aureus	Buffalo	Diseased	India	N/A	21	Milk	81.0	19.0		N/A	Sharma et al., 2020
Staphylococcus aureus	Goat	Diseased	India	N/A	28	Milk	92.9	7.1		N/A	Sharma et al., 2020
Staphylococcus aureus	Sheep	Diseased	India	N/A	6	Swabs	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus	Camel	Diseased	India	N/A	8	Swabs	62.5	37.5		N/A	Sharma et al., 2020
Staphylococcus aureus	Horse	Diseased	India	N/A	3	Swabs	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus (MRSA ST 398)	Various (rabbit, human)	N/A	Italy	2013	7	Swabs	100.0			0.25 - 0.5	Agnoletti et al., 2014
Staphylococcus aureus (MRSA ST 398)	Pig	N/A	Spain	N/A	7	Swabs	S (5 iso)	I (2 iso)		N/A	Lozano et al., 2011
Staphylococcus aureus (MRSA ST 793)	Pig	N/A	Spain	N/A	1	Swabs			R (1 iso)	N/A	Lozano et al., 2011
Staphylococcus aureus (MDR)	Cattle	Diseased	Bangladesh	2017-2018	48	Milk	S			N/A	Salauddin et al., 2020
Staphylococcus aureus	Cattle	Healthy	Bangladesh	N/A	11	Milk	S			N/A	Tanzin et al., 2016
Staphylococcus aureus	Buffalo	Healthy	Bangladesh	N/A	1	Milk	S			N/A	Tanzin et al., 2016
Staphylococcus aureus	Cattle	Diseased	India	N/A	20	N/A	S			N/A	Upadhyay and Kataria, 2009
Staphylococcus aureus	Goat	Diseased	India	N/A	10	N/A	S			N/A	Upadhyay and Kataria, 2009
Staphylococcus aureus	Goat	Healthy	China	N/A	32	Swabs	84.4		15.6	N/A	Zhou et al., 2017
Staphylococcus aureus	Horse	Healthy	Belgium	2010-2011	2	Swabs	S			N/A	Van den Eede et al., 2013

MRSP - Multidrug-resistant *Staphylococcus pseudintermedius*, MRSA - methicillin-resistant *Staphylococcus aureus*, MDR – multidrug resistant, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source.

feces, diffuse erythema, pruritus, and signs of depression in two of the animals in their study following intravenous administration of 15 mg/kg levofloxacin in dogs. High single doses (810 mg/kg) of oral levofloxacin have also been reported to cause gastrointestinal side effects in female rats (Watanabe et al., 1992). Interestingly, the same study found that a much lower single oral dose (50 mg/kg) of levofloxacin in rabbits also caused gastrointestinal issues (reduction in food intake and body weight). Similarly, a toxicological study in broiler birds reported that a dose of 60 mg/kg bodyweight (considered therapeutic) was associated with gastrointestinal and hematological adverse effects, while supra-therapeutic doses caused more severe gastrointestinal and hematological toxicity as well as muscle weakness and loss of body weight (Kumar et al., 2009b). Despite the few reports of overt side effects in animals, molecular studies have found adverse effects of levofloxacin on various tissues, especially with extended dosing regimens. Khan and Rampal (2013) reported a reduction in antioxidant activity in rabbits following 21 days of oral treatment with 10 mg/kg bodyweight levofloxacin. In rats, oral administration of levofloxacin for 4 weeks revealed cytotoxic but not genotoxic effects (Al-Soufi and Al-Rekabi, 2018). Oral administration of levofloxacin for 30 days at doses from 9.37 to 37.5 mg/kg body weight resulted in deleterious effects on the liver, kidney and testes in mice (Ara et al., 2020), however Farid and

Table 4b
Susceptibility of Gram-positive and atypical microorganisms isolated from animals against levofloxacin

Bacteria	Animal species	Health status	Country	Sampling period	N	Sample type	S %	I %	R %	MIC	Reference
Enterococcus spp.	Cattle	N/A	Canada	2018	176	Feces		0.0	0.0	N/A	Davedow et al., 2020
Lactobacillus spp.	Poultry (Indigenous)	N/A	Pakistan	N/A	59	Rectal swabs, feces			81.4	32 - >128	Saleem et al., 2018
Lactobacillus spp.	Poultry (commercial)	N/A	Pakistan	N/A	46	Rectal swabs, feces			97.8	32 - >128	Saleem et al., 2018
Actinomyces bowdenii	Dog	Diseased	USA	N/A	1	Tissue			R	N/A	Sherman et al., 2013
Streptococcus spp.	Cattle	Diseased	India	N/A	46	Milk	89.1	6.5	2.2	N/A	Mohanty et al., 2013
Streptococcus spp.	Buffalo	Diseased	India	N/A	1	Swabs	100.0			N/A	Bhadaniya et al., 2019
Streptococcus agalacticae	Elephants (captive)	Diseased	Germany	2014-2015	25	Swabs	100.0			<1	Eisenberg et al., 2017
Streptococcus agalacticae	Cattle	Diseased	China	2014-2017	133	Milk	18.1	18.1	63.9	N/A	Yang et al., 2020
Streptococcus suis	Pig	Diseased	Japan	2004-2007	16	Tissues	100.0			0.25 - 1	Ichikawa et al., 2020
Streptococcus suis	Pig	Healthy, diseased	Japan	2014-2016	98	Tissues, swabs	100.0			0.5 - 4	Ichikawa et al., 2020
Streptococcus suis	Pig	Healthy	Brazil	2019-2010	260	Swabs	62.3	6.2	31.5	N/A	Soares et al., 2014
Clostridium difficile	Dog (puppy)	Healthy	Spain	N/A	34	Rectal swabs			100.0	>32	Álvarez Pérez et al., 2014a,b
Clostridium difficile	Cattle (beef)	N/A	USA	N/A	94	Feces			100.0	2 - >32	Thitaram et al., 2016
Clostridium difficile	Cattle (dairy)	N/A	USA	N/A	188	Feces			96.8	2 - >32	Thitaram et al., 2016
Clostridium difficile	Pig	N/A	USA	N/A	94	Feces			100.0	2 - >32	Thitaram et al., 2016
Clostridium difficile	Cattle (calf)	Healthy, diseased	Canada	2004	30	Feces			73.0	4 - >32	Rodriguez-Palacios et al., 2006
Clostridium difficile	Cattle	N/A	Slovenia	N/A	103	Feces				<2 - 16	Bandelj et al., 2017
Clostridium difficile	Pig	Healthy	Spain	N/A	41	Rectal swabs			100.0	>32	Álvarez-Pérez et al., 2013
Clostridium difficile	Zebra (*Equus quagga burchellii*)	Healthy	Spain	N/A	4	Rectal swabs			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Goat	Healthy	Spain	N/A	1	Rectal swabs			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Iberian ibex (*Capra pyrenaica hispanica*)	Healthy	Spain	N/A	1	Rectal swabs			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Chimpanzee (*Pan troglodytes troglodytes*)	Diseased	Spain	N/A	1	Tissues			100.0	>32	Álvarez-Pérez et al., 2014b
Bacillus spp.	Buffalo	Diseased	India	N/A	3	Swabs	66.6			N/A	Bhadaniya et al., 2019
Micrococcus spp.	Buffalo	Diseased	India	N/A	9	Swabs	88.8			N/A	Bhadaniya et al., 2019
Corynebacterium spp.	Buffalo	Diseased	India	N/A	11	Swabs	90.9			N/A	Bhadaniya et al., 2019
Mycoplasma bovis	Cattle (beef, dairy)	N/A	China	2008-2011	26	N/A	S			0.5 - 2	Mustafa et al., 2013
Mycobacterium avium subsp.*hominissuis*	Cat	Diseased	Japan	N/A	1	Tissues			R	1	Kanegi et al., 2019

N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source.

Hegazy (2020), found no clinical signs of levofloxacin-induced liver toxicity after oral administration of 40 mg/kg bodyweight in rats for just two weeks (although liver enzymes associated with liver damage and oxidative stress markers were elevated). Finally, some experimental reports and case studies have reported other potential effects of levofloxacin in animals. An experiment by Erden et al. (2001) revealed an anxiety-like effect in rats, and a reduction in sleep in mice. Interestingly, this study also suggested that levofloxacin had analgesic activity in mice. Finally, a case report (Park et al., 2015) reported the development of a corneal plaque containing levofloxacin in a dog, following administration of levofloxacin eye drops for a period of 2 weeks.

4.6. Pharmacokinetics

Levofloxacin pharmacokinetic profiles have been established for different animal species, however these used different analytical techniques for levofloxacin concentration detection (microbiological assay, HPLC with fluorescence detection, HPLC with UV/Vis detection, HPLC/MS), different experimental protocols and different pharmacokinetic modelling approaches. This makes comparing such data challenging. Some authors indicate that the pharmacokinetics of levofloxacin is best described by a two-compartmental pharmacokinetic model (Goudah and Abo-El-Sooud, 2009; Ram et al., 2011; Czyrski et al., 2015), while others applied a non-compartmental approach (Lee et al., 2017; Vercelli et al., 2020; Sitovs et al., 2020). Comparison of the main

pharmacokinetic parameters in mammalian species is presented in Tables 5a and 5b. The fastest clearance was observed in rabbits (Sitovs et al., 2020) and sheep (Patel et al., 2012a), and the longest elimination in cats (Albarellos et al., 2005). Bird pharmacokinetic parameters are presented in Table 6. Here, the fastest clearance was observed in broiler chickens by El-Banna et al. (2013), however other studies on chickens have shown slower clearance values. In particular, Lee et al. (2017) reported the longest elimination in broiler chickens. Of other poultry, Bilgorajska geese had the longest elimination time (Sartini et al., 2020b).

4.6.1. Plasma protein binding

Plasma protein binding of levofloxacin in animals is generally lower than reported in humans (38%; Fish and Chow, 1997). The *in vitro* plasma protein binding of levofloxacin has been assessed in various species (Table 7), with the highest reported plasma protein binding in rats (45.5%, Hurtado et al., 2014), and the lowest in broiler chickens (4.2%, El-Banna et al., 2013). Protein binding was never high enough to significantly affect levofloxacin pharmacokinetics.

4.6.2. Tissue disposition and residues

Table 8 presents levofloxacin disposition in poultry tissues, including suggested withdrawal times. Withdrawal times for registered veterinary

Table 5a
Main levofloxacin pharmacokinetic parameters (±SD) reported in mammals after a single administration[a].

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	T1/2el (h)	Vdss (L/kg)	MRT	F%	Reference
Dog	IV	5.0	0.29 ± 0.09	7.93 ± 1.41		8.75 ± 1.57		Urzúa et al., 2020
Dog	PO	5.0		7.65 ± 1.38		9.37 ± 1.70	72 ± 10	
Dog	IV	15.0	0.15 ± 0.03	6.23 ± 0.91	1.19 ± 0.20	8.31 ± 0.94		Madsen et al., 2019
Dog	PO	23.7		5.84 ± 1.17			104 ± 30	
Dog	IV	2.5	0.11 ± 0.03	7.85 ± 2.30	1.20 ± 0.13			Landoni and Albarellos, 2019
Dog	SC	5.0		7.78 ± 1.55			80 ± 8	
Dog	PO	5.6		6.01 ± 1.32			61 ± 15	
Dog	PO 300mg			4.92 ± 1.94				Yin et al., 2011
Dog	PO 300mg (SR 1)			7.15 ± 2.13			42 ± 5	
Dog	PO 300mg (SR 2)			8.40 ± 1.01			103 ± 4	
Cat	IV	10.0	0.14 ± 0.04	9.31 ± 1.63	1.75 ± 0.42	12.99 ± 2.12		Albarellos et al., 2005
Cat	PO (4 days mean)	10.0		8.39 ± 2.14			86 ± 44	
Giant panda (Ailuropoda melanoleuca)	IM	2.0		5.40 (0.70)[b]				Wang et al., 2021
Giant panda (Ailuropoda melanoleuca)	PO	3.0		7.14 (0.63)[b]				
Rabbit	IV	5.0	0.60 ± 0.18	2.06 ± 0.18	1.37 ± 0.39	2.19 ± 0.83		Sitovs et al., 2020
Rabbit	IM	5.0		2.01 ± 0.24		3.75 ± 1.16	106 ± 28	
Rabbit	SC	5.0		1.80 ± 0.14		3.44 ± 1.31	119 ± 41	
Rabbit	IV (30 min inf)	20.0	1.7 (L/h)	3.99 ± 0.92		3.64 ± 0.76		Czyrski et al., 2015
Rabbit (Meningitis model)	IV (10 min inf)	7.0		7.60 ± 3.50				Destache et al., 2001
Rabbit (Meningitis model)	IV (10 min inf)	10.5		7.00 ± 1.60				
Rabbit (Meningitis model)	IV (10 min inf)	14.0		9.50 ± 3.50				
Guinea pig (Pneumonia model)	IP	10.0		1.00 ± N/A				Edelstein et al., 1996
Rat	IV	7.0	0.21 (L/h)	5.00 ± 1.70	1.20 ± 0.40	6.10 ± 2.70		Hurtado et al., 2014
Rat	PO	100.0		1.76 ± N/A				Dharuman et al., 2010
Rat	IV	3.0				1.66 ± 0.58		Cheng et al., 2002
Mouse	PO	10.0		5.65 ± 0.14		8.46 ± 0.27		Yarsan et al., 2003
Mouse (Toxoplasmosis model)	PO	10.0		4.54 ± 0.50		6.63 ± 0.71		Yarsan et al., 2003

SD – standard deviation, ROA – route of administration, IV – intravenous, IM – intramuscular, SC – subcutaneous, IP – intraperitoneal, PO – oral, BW – body weight, SR – sustained release, inf – infusion, Cl – plasma clearance, T1/2el – half-life of elimination, Vdss – volume of distribution at steady state, MRT – mean residence time, F – bioavailability, N/A – data not available in the reference source.

[a] Unless otherwise noted.

[b] Median value (range).

Table 5b
Main levofloxacin pharmacokinetic parameters (±SD) reported in mammals after a single administration[a].

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	T1/2el (h)	Vdss (L/kg)	MRT	F%	Reference
Cattle (calf)	IV	10.0	0.34 ± 0.01	2.12 ± 0.21	0.98 ± 0.10	2.87 ± 0.31		Kumar et al., 2012
Cattle (calf)	IM	10.0		2.76 ± 0.36		4.72 ± 0.72	63 ± 6	
Cattle (crossbred calf)	PO	20.0		2.99 ± 0.15		4.66 ± 0.14		Kumar et al., 2009a, 2009b
Cattle (crossbred calf; febrile)	PO	20.0		3.05 ± 0.16		5.04 ± 0.14		
Cattle (crossbred calf)	IV	4.0	0.32 ± 0.05	1.61 ± 0.07	0.74 ± 0.03 (Varea)	2.13 ± 0.09		Dumka and Srivastava, 2007
Cattle (crossbred calf)	IM	4.0		3.67 ± 0.40		5.57 ± 0.51	57 ± 12	Dumka and Srivastava, 2006
Buffalo (calf)	IM	3.0		3.27 ± 0.31		5.40 ± 0.59	68 ± 5	Ram et al., 2008
Goat (non-lactating)	IV	2.0	0.46 ± 0.11	4.56 ± 1.24	1.22 ± 0.22			Vercelli et al., 2020
Goat (non lactating)	SC	2.0		5.14 ± 0.57			92 ± 59	
Goat	IV	10.0	0.34 ± 0.05	4.04 ± 0.24	1.89 ± 0.18 (Varea)	5.61 ± 0.49		Ram et al., 2011
Goat (Mastitis model)	IV	10.0	0.35 ± 0.03	5.08 ± 0.18	2.56 ± 0.21 (Varea)	7.77 ± 0.28		
Goat (lactating)	IV	4.0	0.18 ± 0.04	2.95 ± 0.27	0.73 ± 0.22	3.74 ± 1.21		Goudah and Abo-El-Sooud, 2009
Goat (lactating)	IM	4.0		3.64 ± 0.42		5.24 ± 1.12	85 ± 8	
Sheep	IV	2.0	0.19 ± 0.02	4.06 ± 2.41	0.56 ± 0.18	3.24 ± 0.98		Sartini et al., 2020a
Sheep	PO (5 days)	2.0		3.76 ± 1.73		4.25 ± 1.65	115 ± 28	
Sheep	IV	4.0	0.39 ± 0.04	1.82 ± 0.05	0.96 ± 0.08	2.48 ± 0.07		Corum et al., 2020
Sheep	IV	3.0	0.55 ± 0.02	2.38 ± 0.22	0.92 ± 0.08	1.73 ± 0.11		Patel et al., 2012a, 2012b
Sheep	SC	3.0		1.73 ± 0.04		2.67 ± 0.04	91 ± 4	
Sheep	IV	4.0	0.20 ± 0.05	3.29 ± 0.23	0.86 ± 0.23	4.26 ± 0.94		Goudah and Hasabelnaby, 2010
Sheep	IM	4.0		3.58 ± 0.30		5.33 ± 1.05	91 ± 7	
Camel	IV	4.0	0.28 ± 0.03	2.92 ± 0.61	1.01 ± 0.36	3.47 ± 0.81		Goudah, 2008
Camel	IM	4.0		3.47 ± 0.86		5.58 ± 0.94	94 ± 8	
Horse (Stallion)	IV	4.0	0.21 ± 0.18	2.58 ± 0.51	0.81 ± 0.26	3.94 ± 0.61		Goudah et al., 2008
Horse (Stallion)	IM	4.0		2.94 ± 0.78		4.72 ± 0.54	92 ± 13	
Marmoset	PO	40.0		3.90 ± N/A				Nelson et al., 2010
Marmoset	PO (7 days)	40.0		2.30 ± N/A				
Rhesus monkey (Anthrax model)	PO	15.0		2.10 ± 0.12				Kao et al., 2006
Rhesus monkey (Anthrax model)	PO	25.0		1.86 ± 0.28				
Rhesus monkey (male)	PO (C14-labelled)	15.0		1.67 ± N/A				Hemeryck et al., 2006
Rhesus monkey (female)	PO (C14-labelled)	15.0		1.90 ± N/A				

SD – standard deviation, ROA – route of administration, IV – intravenous, IM – intramuscular, SC – subcutaneous, PO – oral, BW – body weight, SR – sustained release, Cl – plasma clearance, T1/2el – half-life of elimination, Vdss – volume of distribution at steady state, MRT – mean residence time, F – bioavailability, N/A – data not available in the reference source.
[a] Unless otherwise noted.

products containing levofloxacin are reported in Table 1.

Multiple pharmacokinetic studies have also reported on the distribution of levofloxacin in the tissues of various mammalian species. In rats, levofloxacin reached its highest concentration (2.31 μg/mL) in prostate dialysate fluid following intravenous administration of 7 mg/kg bodyweight levofloxacin (Hurtado et al., 2014). After a single intravenous administration of 0.5 μmol/kg to rats (0.18 mg/kg), Ito et al. (1999) reported the highest levofloxacin concentration within 3 minutes in the kidney medulla - 10.4 nmol/g (3758 μg/kg), followed by the kidney cortex - 6.2 nmol/g (2241 μg/kg) and the lowest concentration in brain - 0.03 nmol/g (11 μg/kg). Sartini et al. (2020a) investigated the distribution of levofloxacin in several tissues in sheep (muscle, liver, kidney, heart, lung), following intravenous administration of the drug daily for five days. The highest reported concentration of levofloxacin

Table 6
Main levofloxacin pharmacokinetic parameters (±SD) reported in birds after a single administration[a].

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	T1/2el (h)	Vdss (L/kg)	MRT	F%	Reference
Poultry (not specified)	IM (5 days)	10.0		2.97 ± 0.11				Bisht et al., 2018
Chicken (broiler)	IV	5.0	0.38 ± 0.09	6.93 ± 2.94	2.88 ± 1.07	5.37 ± 1.31		Lee et al., 2017
Chicken (broiler)	PO	5.0		8.09 ± 1.71		6.90 ± 0.37	123 ± N/A	
Chicken (broiler)	IV	10.0	0.44 ± 0.01	4.07 ± 0.24	2.36 ± 0.13	5.40 ± 0.26		El-Banna et al., 2013
Chicken (broiler)	PO	10.0		4.24 ± 0.28		6.59 ± 0.44	107 ± 9	
Chicken (Leghorn bird)	IV	10.0	0.25 ± 0.00	3.08 ± 0.05	3.23 ± 0.06	3.57 ± 0.05		Patel et al., 2012b
Chicken (Leghorn bird)	PO	10.0		3.62 ± 0.12		6.41 ± 0.13	72 ± 1	
Chicken (broiler)	IV	10.0	0.25 ± 0.00	3.18 ± 0.07	3.25 ± 0.06	3.69 ± 0.08		Varia et al., 2009
Chicken (broiler)	PO	10.0		3.64 ± 0.15		6.12 ± 0.13	60 ± 2	
Turkey	IV	10.0	0.23 ± 0.03	4.49 ± 0.12	1.31 ± 0.04	5.20 ± 0.30		Aboubakr, 2012
Turkey	IM	10.0		4.60 ± 0.22		6.68 ± 0.17	96 ± 4	
Turkey	PO	10.0		4.07 ± 0.17		6.30 ± 0.13	80 ± 3	
Quail (Japanese)	IV	10.0	0.40 ± 0.03	2.52 ± 0.07	1.27 ± 0.06	2.72 ± 0.09		Aboubakr, 2012
Quail (Japanese)	PO	10.0		2.83 ± 0.30		4.26 ± 0.08	69 ± 2	
Geese (Bilgorajska)	IV	2.0	0.28 ± 0.06	7.39 ± 1.21	1.40 ± 0.28	5.12 ± 0.37		Sartini et al., 2020b
Geese (Bilgorajska)	PO	5.0		6.60 ± 2.46			96 ± 21	
Duck (Muscovy)	IV	10.0	0.41 ± 0.04	2.76 ± 0.10	1.37 ± 0.07	3.34 ± 0.16		Aboubakr and Soliman, 2014
Duck (Muscovy; renal damage)	IV	10.0	0.20 ± 0.02	4.71 ± 0.54	1.18 ± 0.04	6.13 ± 0.76		
Duck (Muscovy)	PO	10.0		2.89 ± 0.09		4.08 ± 0.14	74 ± 2	
Duck (Muscovy; renal damage)	PO	10.0		3.94 ± 0.14		6.83 ± 0.19	72 ± 2	

SD – standard deviation, ROA – route of administration, IV – intravenous, IM – intramuscular, PO – oral, BW – body weight, Cl – plasma clearance, T1/2el – half-life of elimination, Vdss – volume of distribution in steady state, MRT – mean residence time, F – bioavailability, N/A –data not available in the reference source.
[a] Unless otherwise noted.

Table 7
Average levofloxacin plasma protein binding (± SD).

Mammals	Protein binding %	Reference
Dog	23.7 ± 3.8	Madsen et al., 2019
Rabbit	25.0 ± N/A	Destache et al., 2001
Rat	45.5 ± 9.4	Hurtado et al., 2014
Cattle (crossbred calf)	17.0 ± 1.2	Dumka and Srivastava, 2006
Buffalo (calf)	19.1 ± 1.5	Ram et al., 2008
Goat	Range: 23.0 – 34.8	Ram et al., 2011
Goat (lactating)	22.0 ± N/A	Goudah and Abo-El-Sooud, 2009
Sheep	23.7 ± N/A	Goudah and Hasabelnaby, 2010
Camel	23.5 (Range 21.0 – 27.0)	Goudah, 2008
Horse (stallion)	27.8 (Range 20.0 – 29.0)	Goudah et al., 2008
Rhesus monkey	11.2 ± N/A	Hemeryck et al., 2006
Birds	**Protein binding %**	**Reference**
Chicken (broiler)	24.0 ± 5.0	Lee et al., 2017
Chicken (broiler)	4.2 ± 0.5	El-Banna et al., 2013
Turkey	24.3 ± N/A	Aboubakr et al., 2014
Quail (Japanese)	23.0 ± N/A	Aboubakr, 2012

SD – standard deviation, N/A –data not available in the reference source

was in the kidney, and all tissues had detectable levels of levofloxacin 48 hours after the final dose was administered. This study also reported no accumulation of levofloxacin in the plasma or organs. Levofloxacin was found to penetrate better than other fluoroquinolones into the lungs of mice (Klesel et al., 1995) and to accumulate in the lung of guinea pigs (Edelstein et al., 1996). Ocular concentrations reached their highest levels 1 hour post oral administration of 20 mg/kg bodyweight levofloxacin in rabbits (Mochizuki et al., 1994). Interestingly, in this study, ocular concentration was higher in pigmented rabbits compared to albino ones. Finally, after ophthalmic administration, Sakai et al. (2019) reported comparable concentrations in extraocular tissues, eyelid, conjunctiva and cornea.

Given the importance of minimising antibiotic residues in milk for human consumption, levofloxacin distribution into and elimination from the milk has been studied. As a weak organic acid, levofloxacin is expected to rapidly diffuse into the milk (Ram et al., 2008). It is therefore unsurprising that studies have investigated this phenomenon in milk-producing animals. Levofloxacin distribution in goat milk was studied by Goudah and Abo-El-Sooud (2009) and Ram et al. (2011). After the administration of 4 mg/kg bodyweight, Goudah and Abo-El-Sooud (2009) reported milk protein binding of 37% and a good penetration rate from blood to milk after intravenous and intramuscular administration. AUC_{milk}/AUC_{plasma} ratios were 0.81 and 1.01 respectively. Elimination half-life from milk was similar regardless of administration route, and shorter than 4 hours. Interestingly, Ram et al. (2011) reported a longer elimination half-life from milk in mastitic goats (7.5 hours) versus in healthy goats (4.5 hours) after intravenous administration of 10 mg/kg bodyweight levofloxacin, highlighting the importance of considering potential differences in elimination induced by concurrent disease.

4.6.3. Metabolism

Formation of metabolites is negligible in view of levofloxacin antimicrobial activity in humans, with no active metabolites identified. Very limited data is available regarding the metabolic pathways of levofloxacin in animals. Fish and Chow (1997) reported minimal formation of levofloxacin beta-glucuronide (M1, not identified in humans), desmethyl-levofloxacin (M2), and levofloxacin-N-oxide (M3) in rats, dogs and monkeys. Similar results were also reported by Hemeryck et al. (2006) in Rhesus monkeys, with a further two unnamed metabolites also identified. The authors proposed that metabolites were formed directly from levofloxacin by N-demethylation, N-oxidation and glucuronide conjugation. All metabolites were in far lower concentrations than the parent compound.

4.6.4. Bioavailability

Relative bioavailability of levofloxacin is among the highest of all fluoroquinolones, reported as over 100% in multiple studies (Madsen et al., 2019; Sartini et al., 2020a; Lee et al., 2017), and thus considered complete (Tables 5a, 5b, 6). Complete oral bioavailability was reported in sheep (Sartini et al., 2020a), dogs (Yin et al., 2011; Madsen et al., 2019), and chickens (El-Banna et al., 2013; Lee et al., 2017). The lowest oral bioavailability was reported by Yin et al. (2011) after administration of a sustained-release formulation in dogs. Bioavilability following intramuscular and subcutaneuous administration is variable between species, with the range of intramuscular bioavailability being 57-106%, and subcutaneous bioavailability 80-119%. Complete bioavailability following intramuscular and subcutaneous administration has been

Table 8
Tissue disposition and suggested withdrawal times of levofloxacin in poultry.

Species	ROA	Dose (mg/kg)	Dose frequency	Cmax (μg/kg)	Tmax	Tlast	PCO	Tissues analyzed	S WT	Reference
Chicken	PO	10	5 days	1051	0d	10d	Liver	Muscle, liver, gizzard, heart, skin	N/A	Kyuchukova et al., 2013
Chicken (broiler)	PO	10	3 days	9330	2h	9d	Kidney	Muscle, liver, kidney, lung, fat, spleen	>9 days	El-Banna et al., 2013
Chicken	PO	10	5 days	1429	1d	10d	Liver	Muscle, liver	4 days	Ravikumar et al., 2016
Chicken (broiler)	PO	5	Single	657	1h	48h	Liver	Muscle, liver, kidney, lung	N/A	Lee et al., 2017
Chicken	PO	10	28 days	1222	1 d	10d	Liver	Muscle, liver	5 days	Suman et al., 2018
Chicken	PO	20	28 days	2251	1 d	10d	Liver	Muscle, liver	5 days	Suman et al., 2018
Poultry	IM	10	5 days	140	24h	72h	Kidney	Muscle, liver, kidney	N/A	Bisht et al., 2018
Geese (Bilgorajska)	PO	5	Single	642	6h	48h	Liver	Muscle, liver, lung, kidney, heart	90 h	Sartini et al., 2020b

ROA – route of administration, IV – intravenous, IM – intramuscular, PO – oral, Cmax – maximum detected levofloxacin concentration, Tmax - time of maximum detected levofloxacin concentration, Tlast – last detectable levofloxacin concentration, S WT – suggested withdrawal time, PCO – organ or tissue where maximum levofloxacin concentration was detected, N/A – data not available in the reference source

reported in rabbits (Sitovs et al., 2020), with the average value exceeding 90% in multiple studies (Tables 5a and 5b). The lowest parenteral bioavailability was reported by Kumar et al. (2012) in cattle calves – 60% after intramuscular administration. Similarly, the reported range of average oral bioavailability in animals is 42-123%.

4.6.5. Excretion

In Rhesus monkeys, levofloxacin is rapidly excreted unchanged, mainly in urine (58-65%), while minor metabolites (reported above) represented <5% in urine (Hemeryck et al., 2006). In the same study, a minor fraction of administered levofloxacin was excreted in feces (7.4-14.7%) with approximately 1-2% being the parent compound and 4-7% an unknown levofloxacin metabolite. Urinary excretion in cattle and goats has been investigated in several studies (Dumka and Srivastava, 2007; Kumar et al., 2009a, 2009b; Goudah and Abo-El-Sooud, 2009). Dumka and Srivastava (2007) found levofloxacin was detectable in urine 24 hours post intravenous administration in calves, whereas Goudah and Abo-El-Sooud detected levofloxacin in goat urine up to 36 hours after intravenous administration. Goudah and Abo-El-Sooud (2009) also reported urinary levofloxacin concentrations up to 18 times higher than levels in the plasma and milk. Of note for clinicians, Kumar et al. (2009a, 2009b) reported higher urinary excretion of levofloxacin in febrile calves compared to healthy calves.

4.6.6. Pharmacokinetic interactions of levofloxacin with other compounds

The impact of co-administration of levofloxacin with other drugs or natural products on levofloxacin pharmacokinetics has been reported in several research papers. Sucralfate pre-treatment significantly decreased oral levofloxacin absorption in mixed-breed dogs, reducing maximum plasma concentration from 1.95 μg/mL to 0.57 μg/mL, and bioavailability from 72% to 32% (Urzúa et al., 2020). Co-administration of levofloxacin with sunitinib in rabbits (Czyrski et al., 2015) resulted in an increase in the levofloxacin elimination rate constant and decreased its half-life. Corum et al. (2020) reported that co-administration of levofloxacin with either tolfenamic acid or flunixin meglumine resulted in slower levofloxacin elimination. El-Banna et al. (2013) found that pre-treatment of broiler chickens with amprolium and toltrazuril before levofloxacin administration reduced bioavailability and distribution to the internal organs. A number of medications have been reported to not interfere with levofloxacin pharmacokinetics: cyclosporin pretreatment did not affect levofloxacin biliary distribution in rats (Cheng et al., 2002), administration of intramuscular paracetamol did not affect the pharmacokinetics of levofloxacin in cattle calves (Dumka, 2007), and intramuscular ketoprofen did not influence levofloxacin pharmacokinetics in goats (Jatin et al., 2018). Pretreatment with trikatu (mix of plant extracts *Piper nigrum*, *Piper longum*, and *Zingiber officinale*), however, increased levofloxacin bioavailability in the same goat species (Patel et al., 2019).

5. Conclusion

Regardless of its legal status in veterinary medicine around the world, levofloxacin is used in veterinary and human medicine in some of the biggest countries. Whether it is used extralabel in animals or restricted only to human use, does not eliminate the fact that microbial resistance could spread across borders, and the impact of inappropriate levofloxacin use could have enormous repercussions for antimicrobial drug efficacy and global health. This review provides up-to-date information on levofloxacin that will assist veterinary practitioners and scientists to make informed choices regarding appropriate levofloxacin use.

Author contribution

A.S. performed the literature search and review, and wrote the manuscript, I. S. performed searching the literature and contributed to the ideas. M. G. contributed to the idea of this manuscript and has consulted and helped with writing of the manuscript. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interests

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Pharmacokinetic profiles of levofloxacin after intravenous, intramuscular and subcutaneous administration to rabbits (*Oryctolagus cuniculus*)

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ABSTRACT

Levofloxacin pharmacokinetic profiles were evaluated in 6 healthy female rabbits after intravenous (I/V), intramuscular (I/M), or subcutaneous (S/C) administration routes at a single dose of 5 mg/kg in a 3 × 3 cross-over study. Plasma levofloxacin concentrations were detected using a validated Ultra Performance Liquid Chromatography method with a fluorescence detector. Levofloxacin was quantifiable up to 10 h post-drug administration. Mean $AUC_{0\text{-last}}$ values of 9.03 ± 2.66, 9.07 ± 1.80, and 9.28 ± 1.56 mg/h*L were obtained via I/V, I/M, and S/C, respectively. Plasma clearance was 0.6 mL/g*h after I/V administration. Peak plasma concentrations using the I/M and S/C routes were 3.33 ± 0.39 and 2.91 ± 0.56 µg/mL. Bioavailability values, after extravascular administration were complete, - 105% ± 27% (I/M) and 118% ± 40% (S/C). Average extraction ratio of levofloxacin after I/V administration was 7%. Additionally, levofloxacin administration effects on tear production and osmolarity were evaluated. Tear osmolarity decreased within 48 h post-drug administration. All 3 levofloxacin administration routes produced similar pharmacokinetic profiles. The studied dose is unlikely to be effective in rabbits; however, it was calculated that a daily dose of 29 mg/kg appears effective for I/V administration for pathogens with MIC < 0.5 µg/mL.

Keywords: Levofloxacin; rabbits; pharmacokinetics; tears; osmolar concentration

INTRODUCTION

Rabbits have a small role as food-producing veterinary species [1]; however, they are frequently kept as companion animals. Like other small mammals, rabbits are susceptible to a variety of microbial infections, with the most common infective organisms identified as *Pasteurella* spp., *Enterobacteriaceae* spp., *Streptococcus* spp., and *Staphylococcus* spp. [2,3].

Fluoroquinolones, among the most important antimicrobial drugs in veterinary medicine [4], are known for their bactericidal action against a broad spectrum of microorganisms and for their high penetration to tissues and intercellular fluid after systemic administration [5,6].

Conflict of Interest
The authors declare no conflict of interest.

Author Contribution
Conceptualization: Sitovs A, Giorgi M; Data curation: Sitovs A; Formal analysis: Sitovs A; Funding acquisition: Sitovs A, Bandere D, Purvina S; Investigation: Sitovs A, Voiko L, Kovalcuka L, Kustovs D; Methodology: Sitovs A, Giorgi M; Project administration: Sitovs A, Purvina S, Kovalcuka L; Resources: Sitovs A, Bandere D, Purvina S; Software: Sitovs A, Giorgi M; Supervision: Bandere D, Purvina S, Giorgi M; Validation: Sitovs A, Kustovs D, Giorgi M; Visualization: Sitovs A; Writing - original draft: Sitovs A; Writing - review & editing: Sitovs A, Voiko L, Kustovs D, Kovalcuka L, Bandere D, Purvina S, Giorgi M.

The developing threat of antimicrobial resistance due to over- or misuse of antimicrobials [5] can limit the use of existing antimicrobial agents, especially fluoroquinolones in veterinary medicine. Fluoroquinolones are used in veterinary medicine based on strong evidence of their efficacy and the lack of alternative treatment options. Therefore, understanding of drug kinetics and efficacy from experimental modeling of as-yet unapproved drugs for animal use can contribute to the potential use of these drugs in the future. At present, levofloxacin is approved for veterinary use in some countries [7] and might be used in other countries where antimicrobial agent use is not regulated/controlled by local laws. Regardless, the authors do not endorse the extra-label use of levofloxacin; instead, we undertook this study to investigate the potential use of levofloxacin in rabbits as a basis for further research.

Levofloxacin, a third-generation fluoroquinolone, is active against a wide range of Gram-positive and Gram-negative microorganisms and has improved activity, compared to older fluoroquinolones, against streptococci and anaerobes [6,8,9]. The pharmacokinetics (PKs) of levofloxacin has already been established in several domesticated mammalian pets [7,10,11], non-pets [12-14], and birds [15-18]. Moreover, there are several research papers published in recent years that show increased interest in levofloxacin having potential application as an off-label drug for some pet animals (dogs). Pharmacokinetic/pharmacodynamic (PK/PD) indices of fluoroquinolones indicate the effectiveness of this class of drugs [19,20], and they imply that levofloxacin has promise in the treatment of infections in animals [7,11].

In rabbits, the PKs of levofloxacin have been studied only after intravenous (I/V) administration, with limited samples taken following drug administration [21]. Further, the animals in that study were infected with *Streptococcus pneumoniae* for use as a model for meningitis; thus, the kinetics obtained may have been altered due to infective processes. Regardless, the full PK profile of levofloxacin in healthy rabbits has not been established.

I/V administration requires specific administration skills and is unlikely to become routinely used in rabbits as prey species are less tolerant of handling than predator species [9]. In contrast, intramuscular (I/M) and subcutaneous (S/C) routes of administration are suitable for use in rabbits [22] as those methods are easily performed, minimizing handling of and stress to the animal. Thus, I/M or S/C administration in rabbits is more convenient and faster for veterinary practitioners, and, in exceptional cases, the drug could even be administered by the owner. Despite all 3 routes of administration being parenteral, the PKs of each route could differ, affecting the onset and duration of action and bioavailability.

Rabbits have been used as a model to test the effects of eye drops containing fluoroquinolones [23,24]. However, there is no data on the effect on tear production and quality after parenteral administration of levofloxacin or any other fluoroquinolone approved for systemic use in rabbits. The ocular surface requires a tear film to cover the eye surface in order to maintain eye health and function. Dry eye syndrome (DES) occurs as a result of decreased tear production or increased tear film evaporation. DES in humans and animals can lead not only to discomfort but also corneal and conjunctival damage. There are reports in humans and animals showing that systemic use of drugs such as beta-blockers, angiotensin-converting enzyme inhibitors, diuretics, and antimicrobials have ocular side effects, and most of those drugs have been reported to cause DES [25-27]. In addition, there is evidence that systemic administration of other antimicrobial agents—sulphonamides—can decrease tear production in rabbits [26].

The aims of this study were to establish and compare the PK profiles of levofloxacin after single administration via I/V, I/M, and S/C routes in healthy rabbits. Subsequently, the antimicrobial efficacy of levofloxacin was predicted based on the area under the concentration-time curve to the minimal inhibitory concentration (AUC/MIC) ratio obtained, and additionally, the effects on tear quantitative and qualitative parameters were assessed.

MATERIALS AND METHODS

Animals

Six cross-bred female rabbits (*Oryctolagus cuniculus*) (body mass 4.21 ± 0.74 kg), 6 months of age at the beginning of the study, were obtained from the animal facility of the Faculty of Veterinary Medicine, Latvia University of Life Sciences and Technologies. Animals were determined to be healthy based on physical examination, complete blood analysis, and complete ocular examination including biomicroscopy, indirect ophthalmoscopy, and tonometry. Animals received no drug treatment before the study and were allowed to acclimate in their cages for 7 days before the beginning of the study. Rabbits were housed individually in cages under 12-h light/12-h dark cycle with *ad libitum* access to drinking water and hay. Animals were fed standard pelleted food once daily (Purina Professional Rabbit Feed, Purina, USA). The room temperature was maintained at 20°C. Before the study, animals were randomly divided into 3 groups of 2 using research randomizer software. Identifying numbers were placed on each of the animal cages. Animals were weighed immediately before the beginning of the study and before every drug administration period.

Chemicals and reagents

Analytical standard (purity > 98%) levofloxacin and enrofloxacin (used as the internal standard) and tetraethylammonium chloride were purchased from Sigma-Aldrich (USA). Acetonitrile, methanol, sodium dihydrogen phosphate, sodium hydrogen phosphate, chloroform, and isopropanol were of high-performance liquid chromatography grade. A levofloxacin solution (Levoflox 500 mg/100 mL; Claris, India) was used for administration to the animals.

Experimental design and sample collection

A 3-phase, 3-treatment cross-over study design was applied. The experimental protocol was approved by the Animal Ethics Committee of the Republic of Latvia Food and Veterinary Service (Permission 025564). The study was performed according to the guideline for the care and use of laboratory animals. The levofloxacin solution was administered as a single dose of 5 mg/kg body weight. In each phase, doses were administered as follows: I/V route—as a 1 min bolus into the marginal ear vein; I/M route—half of the dose was administered to each of the musculus biceps femoris consecutively (half dose used to avoid muscle damage due to large volume of solution to be administered); S/C route—administered as an injection in the back of the neck region. A fourteen-day washout period was applied, allowing animals to fully clear the drug and to recover from stress related to the experimental procedures. Animal groups for levofloxacin administration were rotated until all 3 phases of the study were completed.

For each phase, a sterile 24G catheter was placed in the central ear artery (for blood collection) and a second one into the marginal ear vein (for I/V drug administration) prior to drug administration on the day of commencement of the experiment. The venous catheter was removed immediately after I/V drug administration while the arterial one remained until

blood collection at 10 h post-administration. Catheters were flushed with heparin containing saline after blood collection, and before any blood collection, the first 0.3 mL of blood were discharged. Blood samples (approximately 0.5 mL) were collected immediately before levofloxacin administration and at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 h post-administration. Blood samples at 24 and 48 h were collected by syringe from the jugular vein. Collected blood was immediately transferred to lithium-heparin containing test tubes, centrifuged at 1000 × *g* for 10 min, and the plasma harvested and stored at −20°C until analysis.

Tear fluid evaluations included tests of tear production and tear film osmolarity. All evaluations were conducted the day before levofloxacin administration to obtain baseline values, and then at 1, 4, 8, 10, 24, and 48 h after each levofloxacin administration.

Baseline Schirmer Tear Test values for tear production were obtained with standardized sterile Schirmer Tear Test I (Eickemeyer, Germany) tips that were inserted under the lower lateral eyelid margin for 1 min. The length of the wet section of the STT tip was immediately measured in millimeters (mm/min). Immediately after the STT result was obtained, STT strips were placed into 1.5 mL polypropylene vials and held at −20°C for further quantification of levofloxacin in the lacrimal fluid. Tear production was also evaluated by applying I-TEAR TEST strips (I-MED Pharma Inc., Canada) into both eyes at the same period post levofloxacin solution administration as that for the STT-based evaluations. A strip was applied to the central lower lid tear meniscus without touching the cornea or conjunctiva in accordance with the manufacturer's instructions. The number of millimeters on the strip reached in 5 sec was obtained (unit: mm/5 sec). Tear film osmolarity was assessed by applying the I-PEN VET device (I-MED Pharma Inc.) immediately after the tear production tests were performed. The I-PEN VET sensor was applied to the palpebral conjunctiva until a sound signal, indicating the end of the measurement, was heard (unit: mOsms/L).

Plasma chromatographic analysis

Levofloxacin concentrations in plasma samples were assessed using a Waters Acquity H Class Ultra Performance Liquid Chromatography system equipped with a fluorescence detector (Waters Corporation, USA). The chromatographic analytical method and the sample extraction procedure were based on those previously described by Lee et al. [16]. Briefly, to 200 µL of plasma, 100 µL of 10 µg/mL internal standard solution in methanol, 800 µL of phosphate buffer solution (pH = 7.0), and 4 mL of chloroform:isopropanol (5:1 v/v) were added. The mixture was shaken by a vertical rotating device (Biosan Bio-RS 24, Latvia) at 30 rotations per minute for 20 min, and then centrifuged at 3,000 × *g* for 10 min at 4°C. Three milliliters of the lower organic layer was transferred into a clean polypropylene tube and evaporated to dryness under a nitrogen stream at 40°C. The dry residue was reconstituted with 200 µL of the mobile phase. One microliter of the resultant solution was injected into the chromatographic system. The chromatographic column used was a Waters Acquity C18 BEH 2.1 × 75 mm with a 1.7 µm particle size (Waters Corporation). The column temperature was maintained at 35°C. The mobile phase was 83% 0.02 M potassium dihydrogen phosphate solution with 0.012 M tetraethylammonium chloride (pH = 2.5) and 17% acetonitrile. The isocratic flow rate was 0.3 mL/min. The fluorescence detector wavelengths were set to 295 nm excitation and 420 nm emission. The sample run time was 5 min.

Chromatographic method validation

Drug-free rabbit plasma was used for both standard curve construction and quality control method validation in accordance with the Guideline on Bioanalytical Method Validation

(EMEA/CHMP/EWP/192217/2009). Drug-free pooled plasma was harvested from all 6 experimental rabbits (2 mL of blood collected) immediately before the beginning of the first phase of the experiment but after the catheters had been placed.

The calibration curve was linear from 0.01 to 10 μg/mL ($R^2 > 0.999$). The levofloxacin recovery from plasma was 96% ± 3.5%. The lower limit of quantification was 0.01 μg/mL. Five level standards of levofloxacin quality controls of 0.01, 0.025, 0.05, 0.5, and 5 μg/mL were used. The between-run accuracy of the method was 1.0%–13.9% and the within-run accuracy was within 15%. The inter- and intra-day precision coefficients of variation were below 5.73% and 5.84%, respectively.

PK analysis

Individual PK parameters were estimated for every animal after treatment using all 3 administration routes. Estimation was performed using non-compartmental analysis and based on visual inspection of the obtained graph (ThothPro Version 1.6.66, Poland). The linear trapezoidal interpolation method was used to calculate the area under the plasma vs. time curve (AUC) after I/V administration, whereas the linear up/log down method was used for the I/M and S/C routes of administration. At least 3 of the last points of the elimination phase of the plasma vs. time curve were used to calculate the elimination constant. The peak plasma concentration (C_{max}), and time to reach peak plasma concentration (t_{max}) were obtained from the data. The bioavailability (F%) was calculated for every single subject as $F\% = (AUC_{I/M \text{ or } S/C}/AUC_{I/V}) \times 100$, and the mean absorption time (MAT) as $MAT = MRT_{I/M \text{ or } S/C} - MRT_{I/V}$. Numerical differences of individual AUC_{0-last} values were lower than 20% of AUC_{0-inf}, and the R^2 of the terminal phase regression line was > 0.85. Extraction ratio (E%) after I/V administration was calculated using the clearance value after I/V administration and the cardiac output value (i.e., E% = clearance/cardiac output ×100), where cardiac output = 180 × body weight$^{-0.19}$ [28].

PK/PD index

Because the levofloxacin concentrations were below the LOQ at 24 h, in order to predict the AUC_{0-24h} and to calculate the PK/PD surrogates, a dose 5 times that administered was modeled. The levofloxacin concentration values for all sampled times from 0.083 h to 10 h post-administration were multiplied by 5. Applying the superposition principle and assuming the same first-order kinetics [29], approximate values of the concentration at 24 h post-administration were calculated for each rabbit for all 3 routes of administration. The non-compartmental PK analysis was re-run to obtain an AUC_{0-24h} value from this adjusted data, and the PK/PD surrogate AUC_{0-24h}/MIC was calculated. Since fluoroquinolones produce a concentration-dependent antimicrobial effect over time [5], a target AUC_{0-24h}/MIC ratio for fluoroquinolones of 72 was used [11].

Drug accumulation prediction

A prediction based on a single administration was used to evaluate the possible accumulation ratio (R) at 12 h dosing intervals (τ). The following formula was used [30]:

$$R = \frac{1}{\left[1 - (0.5)^{\tau/t_{1/2}}\right]}$$

where τ is the dosing interval and $t_{1/2}$ is the half-life of elimination.

MIC breakpoints prediction

Based on the equation $AUC_{0\text{-}24h}/MIC > 72$, the antimicrobial activity breakpoint for the theoretically computed dose of 25 mg/kg for rabbits, a $MIC < AUC_{0\text{-}24h}/72$ was assumed to be effective [11]. The AUC was expressed in terms of the unbound drug; levofloxacin was previously reported to be 25% bound to plasma proteins in rabbits [21].

Theoretical effective daily dose calculation

As fluoroquinolones are antimicrobials that possess concentration/time-dependent effects, a theoretical optimal daily dosage was calculated for all 3 routes of administration based on the following formula [19]:

$$Dose\ per\ day = \frac{\frac{AUC}{MIC} \times MIC \times Cl}{fu \times F} / 24\ \text{h}$$

where $AUC_{0\text{-}24h}/MIC$ is the ratio for optimal efficacy (= 72), Cl = clearance, fu = free fraction of drug in plasma (= 0.75) and F = bioavailability (considered 1 if complete).

Statistical analyses

Statistical analysis was performed using SPSS (version 21.0; IBM Corporation, USA). Most statistical parameters are reported as mean ± standard deviation (SD) values. The exceptions are for plasma half-lives (harmonic means were calculated) and t_{max} (median values are reported). The normality of the data was assessed using the Shapiro-Wilk test. Paired *t*-tests were used to compare the statistical differences for PK parameters with normal data distributions in different administration groups. Where data did not have a normal distribution (e.g., V_{area}/F after I/M or S/C administration), the Wilcoxon test was applied. The *p* values lower than 0.05 were considered to indicate statistical significance.

RESULTS

For all 3 administration routes, the drug was quantifiable in plasma for up to 10 h post-administration of 5 mg/kg.

Animals

All 6 animals received levofloxacin via I/V or I/M routes; however, only 4 completed the S/C administration. In the third phase of the cross-over study, 2 animals were excluded—one animal was excluded because of the inability to fix the catheter in either ear artery. The other animal suffered cramps post I/V administration of levofloxacin and died within 48 h post-administration. Post-mortem examination of this animal showed no respiratory tract, kidney, gastrointestinal tract, or liver abnormalities.

The semilogarithmic plots of mean levofloxacin plasma concentrations (± SD) after the 5 mg/kg single dose via all 3 routes of administration are presented in **Fig. 1**. The mean values of PK parameters obtained (± SD) are reported in **Table 1**. The average $AUC_{0\text{-}last}$ values were 9.03 (± 2.66), 9.07 (± 1.80) and 9.28 (± 1.56) mg*h/L after I/V, I/M, and S/C administration, respectively. Maximum plasma concentration reached 3.33 (± 0.39) and 2.91 (± 0.56) μg/mL after I/M and S/C administrations, respectively. The mean extraction rate after 5 mg/kg I/V administration was 7.2% ± 2.1%.

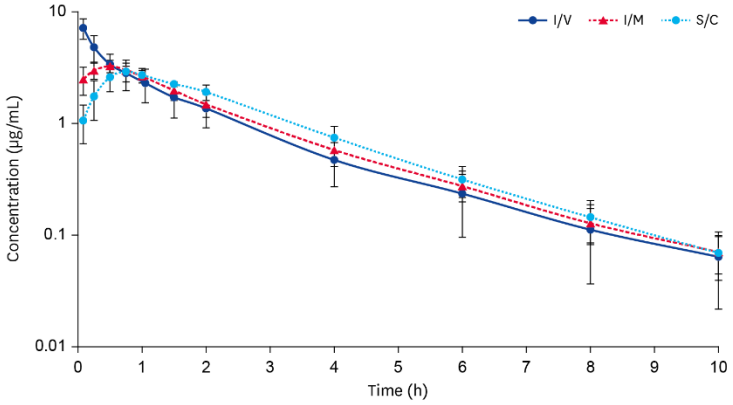

Fig. 1. Semilogarithmic plots of average levofloxacin plasma concentrations in rabbits (error bars represent standard deviations) after I/V (n = 6), I/M (n = 6), and S/C (n = 4) levofloxacin administration of 5 mg/kg bodyweight.
I/V, intravenous; I/M, intramuscular; S/C, subcutaneous.

Table 1. Mean (± SD) pharmacokinetic parameters of levofloxacin in plasma following I/V, I/M or S/C administration to rabbits at a dose of 5 mg/kg bodyweight

PK parameters	Units	I/V (n = 6)	I/M (n = 6)	S/C (n = 4)
AUC_{0-last}	mg*h/L	9.03 ± 2.66	9.07 ± 1.80	9.28 ± 1.56
AUC_{0-inf}	mg*h/L	9.08 ± 2.64	9.07 ± 1.80	9.31 ± 1.50
$AUMC_{0-last}$	mg*h*h/L	22.93 ± 12.46	37.87 ± 18.35*	36.62 ± 17.35
$AUMC_{0-inf}$	mg*h*h/L	23.64 ± 12.17	37.89 ± 18.34*	36.98 ± 16.82
C_{max}	μg/mL	N/A	3.33 ± 0.39	2.91 ± 0.56
C_{first}	μg/mL	7.13 ± 1.47	N/A	N/A
t_{max} MEDIAN	h	N/A	0.50 (0.08–0.75)†	0.75
$t_{1/2\lambda z}$ HM	h	2.06 ± 0.18	2.01 ± 0.24	1.80 ± 0.14
λ_z	1/h	0.34 ± 0.03	0.34 ± 0.04	0.39 ± 0.03
MRT_{0-last} HM	h	2.19 ± 0.83	3.75 ± 1.16*	3.44 ± 1.31
MRT_{0-inf} HM	h	2.27 ± 0.80	3.75 ± 1.16*	3.52 ± 1.25
MAT HM	h	N/A	1.29 ± 0.61	0.45 ± 1.47
Cl	mL/g*h	0.60 ± 0.18	N/A	N/A
Cl/F	mL/g*h	N/A	0.57 ± 0.11	0.55 ± 0.10
V_{ss}	mL/g	1.37 ± 0.39	N/A	N/A
V_{area}/F	mL/g	N/A	1.66 ± 0.34	1.42 ± 0.18
F	%	N/A	105.69 ± 27.50	118.93 ± 40.51

PK, pharmacokinetic; AUC_{0-last}, area under the plasma-concentration time curve from zero to the last quantified sampling point time; AUC_{0-inf}, area under the plasma-concentration time curve from zero extrapolated to infinity; $AUMC_{0-last}$, area under the first moment curve from zero to the last quantified sampling point time; $AUMC_{0-inf}$, area under the first moment curve from zero extrapolated to infinity; C_{max}, maximum plasma drug concentration; C_{first}, concentration at first sample collection point; t_{max}, time of the maximum plasma concentration; $t_{1/2\lambda z}$, half-life of the elimination part of the curve; λz, slope of the elimination part of the curve; MRT_{0-last}, mean residence time from zero to the last quantified sampling point time; MRT_{0-inf}, mean residence time from zero extrapolated to infinity; MAT, mean absorption time; Cl, total plasma clearance; Cl/F, plasma clearance corrected to the bioavailability; V_{ss}, volume of distribution at steady-state; V_{area}/F, volume of distribution corrected to the bioavailability; n, number of experimental animals receiving levofloxacin via the corresponding route of administration; I/V, intravenous; I/M, intramuscular; S/C, subcutaneous; N/A, not applicable; HM, harmonic mean.
*Significantly different from I/V administration ($p < 0.05$); †Range reported.

PK/PD index

The *in silico* obtained AUC_{0-24h} values for the theoretical dose of 25 mg/kg were 44.98 (± 12.54) mg*h/L for I/V administration, 43.11 (± 6.85) mg*h/L for I/M administration, and 43.62 (± 13.65) mg*h/L for S/C administration. The levofloxacin accumulation ratio when administered twice daily (τ =12 h) was predicted to be 1.019 (± 0.006).

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To obtain the AUC/MIC of 72, considering that levofloxacin is 25% bound to plasma proteins, it was calculated that 25 mg/kg of levofloxacin by I/V administration would be effective against pathogens with a MIC < 0.47 μg/mL. In the case of I/M and S/C routes of administration, this dose would be effective against pathogens with a MIC < 0.45 μg/mL. Thus, an effective daily dose against pathogens with a MIC of 0.5 μg/mL was calculated for the I/V administration to be 29 (± 8) mg/kg body weight.

Effects on tear quality

Average tear production observed with STT was 6.4 (± 3.1) mm/min and 7.0 (± 3.1) mm/min, for left and right eyes, respectively (no significant difference, $p = 0.536$). Absolute values varied from 2 to 14 mm/min. No significant changes in tear production were observed among all routes of drug administration within 48 h (data not shown).

Strip meniscometry values, obtained by following the manufacturer's instructions, of 5 mm and higher are considered to indicate normal tear production while smaller values suggest decreased tear production. The average SM measurement results were normal, 6.9 (± 1.3) mm/5 sec and 6.3 (± 1.9) mm/5 sec, for the left and right eyes, respectively (no significant difference, $p = 0.145$). No significant changes in baseline tear production after levofloxacin I/V, I/M, and S/C administration were observed (data not shown).

Tear osmolarity was 324 (± 21) mOsms/L and 331 (± 22) mOsms/L for the 2 eyes prior to drug administration, and the difference was not significant ($p = 0.255$). Mean tear osmolarity decreased in all 3 routes of administration within 48 h after treatment. Changes in tear osmolarity up to 48 h after levofloxacin administration are summarized in **Fig. 2**.

Another area of interest was the quantification of the levofloxacin level in tear fluid in order to evaluate the rationale of ocular infection treatment (conjunctival and corneal infection treatments may be affected by drug distribution in tears). However, the small volume of tear fluid harvested, and the limited sensitivity of the detection method used did not allow quantification of levofloxacin in rabbit tear fluid.

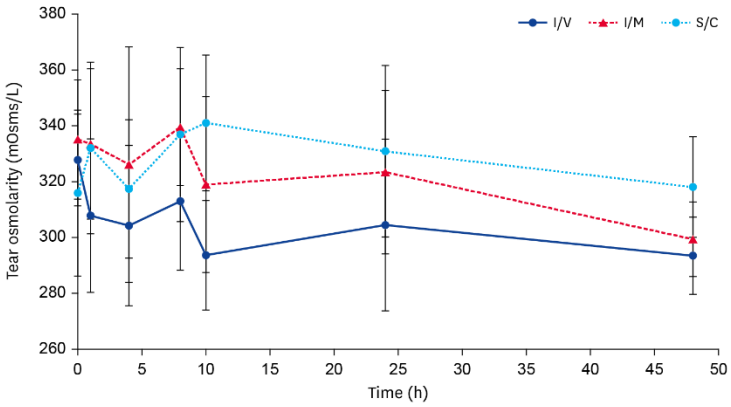

Fig. 2. Changes in tear osmolarity in rabbits after a single 5 mg/kg levofloxacin dose administered via I/V (n = 6), I/M (n = 6), or S/C (n = 4) routes (mean values indicated; error bars represent standard deviation).
I/V, intravenous; I/M, intramuscular; S/C, subcutaneous.

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DISCUSSION

To the authors' best knowledge, this is the first time levofloxacin PK profiles after I/M and S/C administration in healthy rabbits were evaluated, although I/V administration had been examined previously in rabbits infected by *S. pneumoniae*.

The 5 mg/kg dose used in this study was based on the dose used previously in a levofloxacin study involving broiler chicken [16]. This dose is within the range of doses previously used in other mammalian and bird species [10,15,17,18,31]; a dose associated with reduced risks of side effects. Fluoroquinolones are reported to cause tendon damage, seizures, diarrhea in humans, and blindness in cats [4], and Madsen et al. [11] reported side effects, including vomiting, soft feces, and depression, after I/V administration of 15 mg/kg of levofloxacin in dogs. One rabbit died during the current experiment, and the death may be attributed to the stress of the sampling procedures. While necropsy showed no noticeable organ changes in the rabbit, a single I/V dose of levofloxacin in humans has been reported to produce cardiovascular side effects—increased heart rate and QT interval prolongation [32]; thus, cardiovascular effects may also be involved in the lethal outcome in this individual.

All 3 routes of administration (I/V, I/M, and S/C) used in this study produced very similar results for key PK parameters. This could be explained by the fast absorption and rapid distribution of the drug after the extravascular administration routes mimicking the PK profile of the I/V administration. In this study, the AUC values for all 3 routes of administration were similar, and there was complete (calculated over 100%) systemic bioavailability of levofloxacin reported following both I/M and S/C administration. Maximal plasma concentrations for both extravascular routes were reached at around the same time (30–45 min post-administration) and were of similar value (around 3 μg/mL). Similar parallel results were observed for S/C and I/M mean residence times, clearances, and volumes of distribution compared to those for I/V administration. These similarities in PKs suggest that the same drug efficacy should be expected for all 3 routes of administration when levofloxacin is given at a dose of 5 mg/kg. Moreover, previous studies of other fluoroquinolones in rabbits [33,34] and of levofloxacin in other animal species [11,16,35] showed very similar PK profiles after different routes of administration. The levofloxacin terminal plasma half-life appeared to be one of the shortest among the species tested (1.8–2.06 h, depending on the route of parenteral administration).

The volume of drug distribution at a steady-state after I/V administration of 1.37 L/kg suggests moderate penetration of the drug through the biological membranes of the body. This value is within the range reported in avian and mammalian species, 0.92 L/kg in sheep and 2.88–3.25 L/kg in broiler chickens [15,16,35].

Complete bioavailability of levofloxacin after extravascular administration has also been reported in other species [11,13,16]. Interestingly, other fluoroquinolones studied in rabbits after I/M and S/C administration have also shown complete bioavailability, with actual values exceeding 100% [33,36,37]. This may be due to various factors that have already described in the literature [5,6,38], e.g., non-linear clearance. The I/M administration of orbifloxacin, norfloxacin, danofloxacin, and marbofloxacin have all been reported to exceed the 100% bioavailability level [33,34,36,39]. Moreover, S/C ofloxacin, orbifloxacin, and danofloxacin administration also showed complete bioavailability [33,37,40]. These observations indicate that, in general, fluoroquinolones are well absorbed and widely distributed to plasma after I/M or S/C administration in rabbits.

Compared to the study in rabbits infected with *S. pneumoniae* [21], the AUC values of levofloxacin were much lower (at least twice corrected to the dose administered) in the present study. The plasma terminal half-lives of the drug were at least 3 times longer than that observed in our study. These differences might be due to differences in rabbit breed (New Zealand white vs. cross-bred in this study), size of the animals in the 2 studies (2–3 kg vs. 4.2 kg in this study) and the provision of other drugs (e.g., anesthetic administration in [21]). Additionally, the presence of infection may have slowed the elimination of the drug from the body in a manner similar to that observed in a PK study of marbofloxacin in infected rabbits [39].

The AUC values reported for rabbits appear to be the lowest among the other species studied, taking into account the administered dose differences. This might be related to the rapid elimination of the drug from the rabbit body. The average plasma clearance of levofloxacin was 0.6 mL/g*h with some variability among the experimental animals. This is the highest clearance rate thus far reported in all previous mammalian and avian species studied, except sheep, which had similar reported clearance (0.55 mL/g*h [35] vs. 0.6 mL/g*h in rabbits) and half-life of elimination (2.38 h vs. 2.06 h in rabbits) values. However, another study in sheep [14] showed a lower clearance of 0.2 mL/g*h and a longer elimination half-life (3.3 h), but that study was performed using sheep with a body mass almost twice as large, possibly, resulting in slower drug elimination. The high rate of elimination in rabbits may be due to their high cardiac output and heart rate [41]. Higher clearance in rabbits is observed after administration of other fluoroquinolones; orbifloxacin, norfloxacin, danofloxacin, and moxifloxacin are cleared even faster than levofloxacin with clearance values of 0.9, 0.8, 0.8, and 0.8 mL*g/h, respectively [33,34,36,37]. These results indicate that parenteral fluoroquinolone administration in rabbits will require frequent dosing. Alternatively, the route of administration could be changed to consider practitioners' convenience and/or reduction of the handling stress of the infected animal.

A low extraction ratio (around 7%) may indicate that levofloxacin is not fully metabolized and may be excreted unchanged by the kidney [5,6]. This suggests the use of orally administered dosage forms [28]. Although extraction ratio values were not computed in other species in which levofloxacin PKs were established, we calculated approximate extraction ratios for the above-mentioned studies. Low levofloxacin extraction ratios were predicted in cats, dogs, and rabbits (around 2%) based on the clearance and mean animal body weights reported by Albarellos et al. [10], Landoni and Albarellos [7], Madsen et al. [11] and Destache et al. [21]. In food-producing animals, the levofloxacin extraction rate is also low. Based on data provided by Goudah and Abo-El-Sooud [13], Goudah and Hasabelnaby [14], and Patel et al. [35], the authors have calculated average extraction levels for goats, sheep, and camels of 3.2%, 3.9%, and 9.5%. The estimated extraction ratio values in all of the animal species investigated indicate similar drug elimination abilities among the species.

As the elimination half-life of levofloxacin for all 3 routes of administration was short, frequent administration, which is potentially stressful to the animal, would be required. The authors, therefore, do not suggest than any of these parenteral routes are suitable for regular clinical use of levofloxacin in the studied dosage form. While the therapeutic efficacy of fluoroquinolones may be inferred through PK/PD assessment and the use of the AUC/MIC ratio, the low AUC value and the inability to quantify levofloxacin in rabbit plasma at 24 h post drug administration resulted in the inability to perform these surrogate calculations based on our experimental data. Based on our results, a dose of 5 mg/kg of levofloxacin is unlikely to produce a therapeutic effect in rabbits. Our calculated effective daily dose for

levofloxacin, based on an *Enterobacteriaceae* MIC value of 0.5 μg/mL reported in dogs [11], was 29 (± 8) mg/kg, based on a plasma protein binding value of 25% [21]. The estimate is in agreement with the oral dose of 25 mg/kg in dogs to attain similar PK/PD therapeutic targets. In rabbit management, the oral route for drug administration (in medicated feed or water) is the most common one used. Levofloxacin is reported to have complete oral bioavailability in 2 other mammalian species; dog (104 [± 30]%) [11] and cat (86 [± 43]%) [10]. If this oral trend in oral bioavailability is similar in rabbits, the effective daily dose of levofloxacin reported in the current study could be added to pelleted rabbit food or drinking water. However, as infected animals may lose their appetite while maintaining water intake, we suggest the daily dose could be prepared in 50–100 mL of drinking water (i.e., the average daily water intake of rabbits) [42].

This study is the first to investigate the effect of systemic administration of levofloxacin on ocular parameters. The high variability in the qualitative parameters of tears between individual animals before and after treatment with levofloxacin made identification of trends difficult. The authors suggest that the dose may have been too small or a single administration insufficient to produce any discernible effects on tear production. The basal level of the tear production assessed with STT method (7 [± 3] mm/min) was slightly higher than those reported for English angora rabbits and Dutch rabbits (5.4 and 4.6 mm/min, respectively) [43]. Regardless, tear osmolarity appeared to decrease slightly but significantly ($p = 0.002$) at 48 h after drug administration. The authors, therefore, suggest that levofloxacin administration at 5 mg/kg is unlikely to cause major changes in the qualitative and quantitative properties of tears. However, studies with multiple-dose administration and a larger number of animals are warranted.

In conclusion, a levofloxacin dose of 5 mg/kg is unlikely to be effective in rabbits. Moreover, a single administration of that dose is unlikely to have any effect on tear parameters. Based on our calculations, a daily dose of 29 mg/kg may be effective for I/V administration of levofloxacin, but further PK/PD assessments are required to determine its effects.

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Levofloxacin pharmacokinetics and tissue residue concentrations after oral administration in Bilgorajska geese

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ABSTRACT

1. The aim of this study was to assess the pharmacokinetics of levofloxacin, a third-generation fluoroquinolone antimicrobial drug, in geese (n = 26) after either single intravenous or oral administration, and to evaluate the depletion profile in goose muscle, heart, liver, kidney and lung after a single oral dose.
2. The pharmacokinetic study involved 16 geese which were randomly divided into two groups (n = 8/group), the first received levofloxacin (2 mg/kg) intravenously while the second was treated with orally (5 mg/kg). The tissue depletion study involved 10 geese which were dosed orally (5 mg/kg) and two animals were killed at different time-points in order to collect the selected tissues. Levofloxacin was quantified in all the matrices tested by a validated high-performance liquid chromatography (HPLC) method, using a spectrofluorimetric detector. The pharmacokinetics were analysed using a non-compartmental model.
3. Plasma concentrations were quantified after up to 24 h in animals administered intravenously and up to 48 h after oral treatment. Levofloxacin was rapidly absorbed after oral administration (T_{max} = 0.38 h) showing high bioavailability (95.57 ± 20.61%). The drug showed a moderate volume of distribution (1.40 ± 0.28 ml/g) and rapid clearance (0.28 ± 0.06 ml/g/h). No statistical differences in estimates were found between the two different administration methods ($P > 0.05$). Drug residues were highest at 6 h and decreased constantly up to 48 h in all the selected tissues. Liver and kidney had the highest levofloxacin concentrations.
4. According to the pharmacokinetic/pharmacodynamic surrogate index (AUC/MIC) the levofloxacin dose regimen (after oral administration) used in the present study could be active against bacteria at a minimum inhibitory concentration (MIC) > 0.24 μg/ml in geese. In addition, drug accumulation in the liver might be controlled using an estimated preliminary withdrawal time of 90 h.

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Introduction

Geese are often considered wild birds, but they were domesticated a long time ago for their eggs, meat and feathers (Heikkinen 2017; Honka et al. 2018). Waterfowls' meat and eggs have high nutritional quality and geese breeding is increasing all over the world, especially in Europe and Asia (Buckland and Guy 2002). Almost 60 different geese breeds exist, with many located in Eastern Europe (Buckland and Guy 2002). The Bilgorajska goose (*Anser anser domesticus*), the subject of the present study, is a primitive breed from north-eastern Poland (Bilgoraj region) and is actively preserved because of its genetic significance (Książkiewicz 2006).

The health and productive performance of commercial geese is supported *via* modern pharmaceutical management and facilities, nutritional practices and genetic improvement. Infections, caused by pathogens such as *Mycoplasma spp.* or *Pseudomonas spp.*, are common in geese, chickens, turkeys, ducks and ostriches (De Vos et al. 2009; Stipkovits and Szathmary 2012). These pathogens can infect eggs, destroy embryos and, consequently, lead to a significant economic loss (De Vos et al. 2009; Stipkovits and Szathmary 2012). Thus, poultry health is an important factor that constantly requires new protocols in pathogen prevention, control and treatment.

Fluoroquinolones are antimicrobial agents frequently used in poultry production (Gouvêa et al. 2015). There is increasing concern about the uncontrolled and inappropriate use of antibacterial drugs in animal species, and particularly in production animals. Overuse may lead to the transmission of resistant bacteria to humans through meat and eggs, and spread in the environment, compromising human health (EFSA and ECDC 2014; WHO, and AGISAR 2017). An optimal dosing regimen based on a thorough knowledge of the pharmacokinetic profile of any antimicrobial drug can help to minimise the risk of resistance development. Levofloxacin, a third-generation fluoroquinolone, is not yet registered for veterinary species in Europe and the US, but is used to treat animal disease in a small number of countries (e.g. South America (Landoni and Albarellos 2018; Floxaday, Ranbaxy, Argentina). Even where not approved for veterinary use, veterinarians might prescribe the generic form developed for human purposes because of better antimicrobial activity and/or low cost compared to other formulations (Madsen et al. 2019). It is worth emphasising that the aim of this study was not to promote the extra-label use of levofloxacin, but to provide a preliminary investigation on the potential effectiveness of this antimicrobial in geese, as a basis for further studies.

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Levofloxacin is the S(-)-enantiomer of ofloxacin and has excellent broad-spectrum activity against Gram-positive, Gram-negative and anaerobic bacteria as well as atypical pathogens. It is active especially against *Streptococcus pneumoniae*, most Enterococci, *Enterobacteriaceae, Escherichia coli, Klebsiella, Proteus, Pseudomonas, Bacteroides, Clostridium, Haemophilus, Moraxella, Legionella* and *Mycoplasma spp.* (Langtry and Lamb 1998). Levofloxacin pharmacokinetics have been investigated in ducks (Aboubakr and Soliman 2014), chickens (Varia et al. 2009; Patel et al. 2012; Kyuchukova et al. 2013; Lee et al. 2017), quail (Aboubakr 2012) and turkeys (Aboubakr et al. 2014). There are no studies regarding the pharmacokinetics or the tissue disposition of levofloxacin in geese.

The aims of this study were (1) to assess the pharmacokinetics of levofloxacin in Bilgorajska geese after a single intravenous (IV) (2 mg/kg) or oral (PO) (5 mg/kg) administration and (2) to evaluate the drug residue concentrations in selected tissues (muscle, heart, liver, kidney, lung) after a single 5 mg/kg *per os* levofloxacin administration.

Materials and methods

Chemicals and reagents

Levofloxacin and the internal standard (IS) enrofloxacin powder with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). High performance liquid chromatography (HPLC)-grade acetonitrile, methanol, trichloromethane and isopropanol were procured from Merck (Kenilworth, NJ, USA). Tetraethylamine was obtained from Sigma-Aldrich (St Louis, MI, US). Orthophosphoric acid, sodium dihydrogen phosphate and potassium hydrogen phosphate were purchased from Carlo Erba Reagents (Milan, Italy). Deionised water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany).

Experimental design

The experiment was approved by the Institutional Animal Care and Use Committee of the University of Life Sciences (Lublin, Poland) and carried out in accordance with the European law (2010/63/UE). There were two parts of the study, pharmacokinetic and a tissue depletion.

Geese were supplied by a local farm (Majątek Rutka, Puchaczów, Poland). Their health status was evaluated based on a complete physical examination by a veterinarian before the beginning of the study, and through daily observation of behaviour and appetite. Geese were acclimatised for one week in their new environment before the beginning of the trial, and a ring with an identity code was applied to the left leg for easy identification. Birds were housed in a 60 m^2 enclosed area with an indoor shelter of 8 m^2. Animals were allowed to graze freely during the day and were fed a balanced, drug-free pelleted diet (Purina duck feed pellets, Purina Animal Nutrition, Gray Summit, MO, USA) twice a day and water was supplied *ad libitum*. No pharmacological treatment was received by the birds before the experiment.

The pharmacokinetic trial involved 16 healthy male Bilgorajska geese (body weight (BW), 3.4–4.9 kg; age, 3–4 years) which were randomly divided into two subgroups (n = 8/group). Sub-group 1 received a single IV dose (2 mg/kg) of levofloxacin (levofloxacin TEVA 5 mg/ml; Teva Pharmaceutical, Hungary) into the left brachial vein using a sterile 26-gauge 1.75 cm needle. The geese in sub-group 2 were given a single oral dose (5 mg/kg) of levofloxacin. The oral doses were prepared by grinding, homogenising, and partitioning the marketed drug (levofloxacin ACCORD 250 mg/tablet; Accord Healthcare Limited, UK) and dosed relative to the BW of each bird. The correct weight of the solid formulation was dissolved in water and administered *via* crop gavage using a rounded tip metal catheter 3 h after being fed. Blood samples (1 ml) were collected in vacutainer lithium heparin tubes (BD, Vaud, Switzerland) from a 24-gauge catheter inserted immediately before the experiment in the right brachial vein at 0, 5, 15, 30, 45 min and 1, 1.5, 2, 4, 10, 24, 34, and 48 h after IV and at 15, 30, 45 min and 1, 2, 4, 6, 8, 10, 12, 24, and 48 h after the last drug administration after *per os* (PO) treatment. After 12 h, the catheter was removed, and blood was collected from the left brachial vein directly with a 24-gauge syringe. The catheter was cleaned by flushing with 1 ml of 0.9% saline with the addition of 10 IU/ml heparin at each collection timepoint. For each blood collection, the first 0.2 ml of blood was discarded. Tubes were centrifuged at 1500 x *g* and the harvested plasma was stored at −20 °C until analysis within 30 d of collection.

The tissue depletion trial involved 10 geese which were given an oral dose (5 mg/kg) of levofloxacin, as described for sub-group 2. Two animals were humanely killed by stunning and exsanguination at 6, 10, 24, 34 and 48 h after treatment. Approximately 4 g of muscle, heart, liver, lung and kidney were collected and stored at −20 °C until further analysis.

Drug extraction procedure

The procedure was validated for plasma and all tissues collected from the geese, according to Lee et al. (2017), with slight modifications. An aliquot (0.2 ml) of plasma was added to 0.1 ml of IS (0.1 µg/ml) solution in methanol and 0.8 ml of 0.1 M phosphate buffer at pH 7.1. After the addition of 6 ml of a mixture of trichloromethane and isopropanol (5:1 v/v%), the samples were shaken at 60 oscillations/min for 10 min and centrifuged at 4000 x *g* for 5 min. Then 5 ml of the organic layer was transferred into a clean tube and dried at 40 °C under a nitrogen stream. The residue was dissolved in 0.2 ml of mobile phase, vortexed and an aliquot (50 µl) was injected on to the HPLC system.

Liver, kidney, lung, heart and muscle samples were thawed and immediately dissected into small pieces (Sartini et al. 2020). An aliquot of 1 g per tissue was placed into 5 ml plastic tubes containing 3 ml of homogenisation solution (0.1 M phosphate buffer at pH 7.1). The suspension was homogenised for approximately 40 s and then 0.2 ml were processed, as described for the plasma samples.

HPLC instrumentation

The HPLC was an LC system (Jasco, Japan) consisting of a high-pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus), auto sampler (model AS 950), and Peltier system (model CO-4062). The injection loop volume was set at 50 µl. Data was processed using the CromNav 2.0 software (Jasco, Inc.). The chromatographic separation assay, modified from Lee et al. (2017), was performed using a Gemini analytical column (250 × 4.6 mm inner diameter, 5 µm particle size, Phenomenex, Torrance,

California, USA) at 15°C. The mobile phase consisted of acetonitrile: aqueous solution (20:80 v/v%) at a flow rate of 1 ml/min. The aqueous solution consisted of potassium dihydrogenphosphate (0.02 M), phosphoric acid (0.006 M) and tetraethyl amine (0.012 M) in water (pH = 4.0). Excitation and emission wavelengths were set at 295 and 490 nm, respectively.

Validation of the analytical method

The quantitative HPLC method was fully validated for each tissue (liver, kidney, lung, heart and muscle) and plasma in terms of linearity, intra-day and inter-day precision, recovery, limits of detection (LOD) and quantification (LOQ), according to the EMA guidelines (Anonymous 2012).

Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n = 3) of samples from the control geese spiked with levofloxacin at concentrations of 0.005, 0.01, 0.1, 0.5, 1, 5 µg/ml. The intra- and inter-day precision was calculated after analysis of six plasma and tissue samples spiked with levofloxacin at three different concentrations (0.005, 0.1 and 5 µg/ml) with the same instrument and the same operator on the same and on different days, respectively. Precision was calculated and expressed as the coefficient of variation (CV%). The extraction recovery experiment was carried out by analysing samples spiked with the same concentration (0.005, 0.1 and 5 µg/ml) by comparing the response (measured as area) of high, middle, low standards and the IS spiked into blank goose plasma and tissues (control), to the response of equivalent standards. Recovery was expressed as mean ± standard deviation (SD). The LOD was estimated as the plasma and tissue drug concentrations that produced a signal to noise ratio of 3 and LOQ was determined as the lowest plasma concentration that produced a signal to noise ratio of 10.

Pharmacokinetic analysis and statistical analysis

Levofloxacin plasma concentration was modelled for each subject using a non-compartmental model using ThothPro 4.3.0 v software (www. thothpro.com, Gdansk, Poland). The maximum plasma concentration (C_{max}) and time to reach the C_{max} (T_{max}) were determined directly from the concentration *vs* time curves. The elimination half-life ($t_{1/2}\lambda z$) was calculated using least squares regression analysis of the concentration-time curve, and the area under the curve (AUC) was calculated by linear log trapezoidal and the linear-up log-down rule was applied to the final concentration-time points for both IV and PO administration, respectively. From these values, the volume of distribution at steady state (Vss = dose x $AUMC/AUC^2$), mean residence time (MRT = AUMC/AUC), and systemic clearance (Cl = dose/AUC) were calculated. Pharmacokinetic estimates were calculated only if the individual value of $AUC_{rest\%}$ was lower than 20% of $AUC_{(0-\infty)}$ and the square of coefficient of determination (R^2) of the terminal phase regression line was >0.85.

Absolute oral bioavailability (F) was calculated using the following formula:

$$F(\%) = \frac{\text{AUCPO individual} \times \text{dose IV}}{\text{AUCIV avarage} \times \text{dose PO}} \times 100$$

A naïve pooled-data approach, using a non-compartmental analysis (Pouplin et al. 2016), was used to calculate the pharmacokinetic parameters for levofloxacin in all tissue samples.

The penetration of levofloxacin into each tissue was determined by comparing the AUC ratios between tissues and plasma ($AUC_{tissue}/AUC_{plasma}$) after PO administration (Sartini et al. 2019). Levofloxacin concentration in the selected tissues were used to calculate preliminary withdrawal times using the software WT 1.4, developed by the European Medicines Agency (Anonymous 2018). The withdrawal time was established as being the time when the upper-one sided tolerance limit (99%) with 95% confidence interval (CI) was below the maximum residue limit of 0.1 µg/g levofloxacin, which reflected the maximum residue limit (MRL) for many fluoroquinolones in poultry liver (Anonymous 1997, 1999, 2002). The pharmacokinetic parameters were normally distributed (tested by Shapiro–Wilk test) and mean values were compared between the two routes of administration using unpaired *t*-tests using GraphPad Prism v 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Analytical method validation

The validated analytical method showed a good linearity in the range of 0.005–5 µg/ml for every matrix considered in the present study (Table 1). The main results from the analytical method validation in plasma and all tissues selected are reported in Table 1.

Pharmacokinetic results

The semilogarithmic plasma concentration *vs* time curves are shown in Figure 1 after IV and PO administration of a single dose of levofloxacin at 2 mg/kg and 5 mg/kg, respectively.

Plasma levofloxacin concentrations were quantifiable up to 24 h in birds administered intravenously, and up to 48 h after

Table 1. Results of the validation of the HPLC assay used for levofloxacin quantification in plasma and different tissues of Bilgorajska geese.

Parameter	Unit	Plasma	Muscle	Heart	Liver	Lung	Kidney
Equation		y = 2.5446x – 0.0611	y = 0.1113x – 0.0029	y = 0.1356x – 0.0035	y = 0.1894x – 0.0079	y = 0.1722x – 0.0099	y = 0.1454x – 0.0063
R^2		0.999	0.998	0.997	1	0.998	0.996
Inter-day CV	%	5.6	6.1	5.9	6	8.9	7.2
Intra-day CV	%	6.9	10.9	9.6	7.4	10.6	9.9
Recovery	%	96 ± 5	94 ± 10	95 ± 8	98 ± 3	93 ± 8	91 ± 9
LOD	µg/ml	0.001	0.001	0.001	0.001	0.001	0.001
LOQ	µg/ml	0.005	0.005	0.005	0.005	0.005	0.005

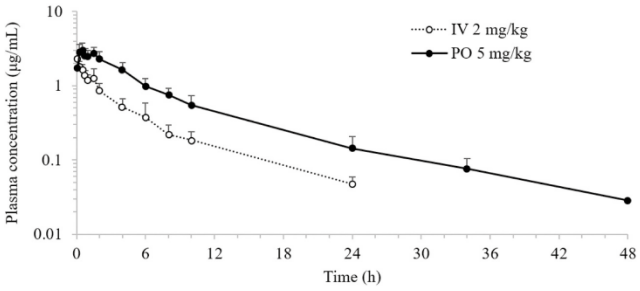

Figure 1. Semilogarithmic plasma levofloxacin concentrations *vs* time curve following IV (··○··, $n = 8$) and PO (–●–, $n = 8$) administration to Bilgorajska geese at a dose of 2 mg/kg BW and 5 mg/kg BW, respectively.

Table 2. Mean (±SD) pharmacokinetic parameters of levofloxacin in plasma following IV (2 mg/kg BW, $n = 8$) or PO (5 mg/kg BW, $n = 8$) administration to Bilgorajska geese.

		IV (2 mg/kg)		PO (5 mg/kg)	
Parameter	Unit	*Mean*	*SD*	*Mean*	*SD*
$AUC_{(0-t)}$	µg*h/ml	7.59	1.77	17.24	4.86
$AUC_{(0-inf)}$	µg*h/ml	8.11	1.76	19.37	4.18
$MRT_{(0-t)}$	h	5.12	0.37	5.71	2.48
$MRT_{(0-inf)}$	h	7.08	0.97	7.65	2.17
λz	1/h	0.10	0.02	0.12	0.05
$t_{1/2}λz$	h	7.39	1.21	6.60	2.46
Vss	ml/g	1.40	0.28	N/A	N/A
Cl	ml/g*h	0.28	0.06	N/A	N/A
Vss/F	m/g	N/A	N/A	1.63	0.49
Cl/F	ml/g*h	N/A	N/A	0.31	0.085
C_{max}	µg/ml	N/A	N/A	3.20	0.65
T_{max}†	h	N/A	N/A	0.38 (0.25–1.5)	
F	%	N/A	N/A	95.57	20.61

$AUC_{(0-t)}$ = area under the curve from 0 h to last time collected samples, $AUC_{(0-inf)}$ = area under the curve from 0 h to infinity, $MRT_{(0-t)}$ = mean residence time from 0 hour to last time collected samples, $MRT_{(0-\infty)}$ = mean residence time from 0 h to infinity, λz = terminal phase rate constant, $t_{1/2}λz$ = terminal half-life, Vss = volume of distribution, Cl = plasma clearance, Vss/F = volume of distribution normalised for F, Cl/F = plasma clearance normalised for F, C_{max} = peak plasma concentration, T_{max} = time of peak concentration, F = bioavailability. †Median value and range.

PO treatment. The slope of the elimination phase appears to be similar for both routes of administration (Table 2).

Table 2 shows the main pharmacokinetic parameters for levofloxacin in geese. Levofloxacin was absorbed rapidly after PO administration displaying a high bioavailability. The drug showed a moderate volume of distribution and a fast clearance. The half-life was not statistically different between the two routes of administration. If normalised for the dose, C_{max} and AUC were not statistically different between the two different administration methods ($P > 0.05$).

Residual tissues analysis results

Results from tissue residue analysis are displayed in Figure 2 as semilogarithmic plots of tissue concentrations *vs* time curves.

Drug residues were highest at 6 h and decreased constantly, remaining over the LOQ up to 48 h (last time-point of collection) in all selected tissues. Liver samples had the highest levofloxacin concentration, followed by kidney samples (Table 3). The pharmacokinetic parameters, calculated by the naïve pooled-data approach for each tissue, are shown in Table 3.

Discussion

The geese did not show any adverse effects during or after drug treatments. The dose was chosen on the basis of a previous study on chickens (Lee et al. 2017). This is the first study which dealt with the pharmacokinetics of levofloxacin in geese. The drug showed a moderate half-life (7.39 h) comparable with results from chickens (6.93 h, Lee et al. 2017), but was longer than in ducks (2.76 h), with a slower Cl (geese, 0.28 ml/g*h; ducks, 0.41 ml/g*h). The Vss in geese (1.40 ml/g) was in line with the value found in ducks (1.37 ml/g). Levofloxacin showed higher AUC (7.59 µg*h/ml), if normalised for dose, than values reported in ducks (4.89 µg*h/ml) and chicken (5.09 µg*h/ml) (Aboubakr and Soliman 2014; Lee et al. 2017). Species specific differences, such as variations in metabolic pathways, plasma protein binding or differences in absorption processes, may have caused these variances. After oral administration, levofloxacin showed faster (T_{max}) and higher (C_{max}) absorption in geese than ducks, turkeys and chickens (Varia et al. 2009; Patel et al. 2012; Aboubakr et al. 2014; Aboubakr and Soliman 2014; Lee et al. 2017). The different formulations administered, variability in experimental design, climatic conditions or feed management might have contributed to such differences. Levofloxacin's oral bioavailability is high in avian species in general (ducks, 73.6%; chickens, 59.5%; leghorn hens, 71.6%; turkeys, 79.9%), but is highest in geese (95.6%), suggesting that the oral route is an appropriate route of administration in birds, and especially geese (Varia et al. 2009; Patel et al. 2012; Aboubakr and Soliman 2014; Aboubakr et al. 2014).

Fluoroquinolones are drugs that act in a concentration-time dependent manner (Forrest et al. 1993), and the ratio of AUC/MIC is considered the pharmacokinetic/pharmacodynamic (PK/PD) index to predict their antimicrobial effects (Turnidge 1999). It has been proposed that a value of 72 for fluoroquinolones can indicate maximum clinical effect in dogs (Madsen et al. 2019). The MIC of levofloxacin has not yet been determined for bacteria isolated from geese. Regarding the $AUC_{(0-24)}$ value obtained in the present study after oral administration (5 mg/kg), levofloxacin in geese appeared be effective against bacteria at a MIC <0.24 µg/ml. For the MIC against *E. coli* isolated in broilers (0.125 µg/m, Lee et al. 2017), a AUC/MIC ratio of 136 was obtained, which suggests that the dose regimen in the present study might be effective in geese. Levofloxacin's plasma protein binding has not been evaluated in geese, but has resulted

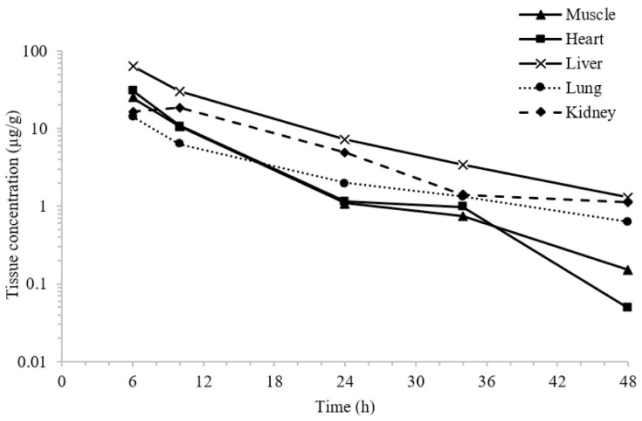

Figure 2. Levofloxacin concentrations (logarithmic scale) in muscle, heart, liver, lung, and kidney following PO administration to Bilgorajska geese (n = 2/timepoint) at a dose of 5 mg/kg BW.

Table 3. Pharmacokinetic parameters calculated by the naive pooled-data approach for levofloxacin in different tissues following oral administration to Bilgorajska geese at a dose of 5 mg/kg BW.

Parameter	Unit	Muscle	Heart	Liver	Lung	Kidney
$AUC_{(0\text{-}t)}$	µg*h/ml	218.72	249.8	687.94	165.26	329.51
$MRT_{(0\text{-}t)}$	h	10.41	9.94	12.56	14.31	13.58
$t_{1/2}\lambda z$	h	8.25	5.07	9.68	14.17	11.84
C_{max}	µg/ml	24.95	30.55	64.2	14.13	18.64
T_{max}	h	6	6	6	6	10
$AUC_{tissue}/AUC_{plasma}$		11.87	13.56	37.35	8.97	17.89

$AUC_{(0\text{-}t)}$ = area under the curve from zero to 48 h; $MRT_{(0\text{-}t)}$ = mean residence time zero to 48 h; $t_{1/2}\lambda z$ = terminal half-life; C_{max} = maximum concentration; T_{max} = time at maximum concentration $AUC_{tissue}/AUC_{plasma}$ = area under the curve ratio of tissue:plasma.

in a low percentage (=25%) in broilers (Lee et al. 2017) and may be considered negligible for the PK/PD surrogate calculation. However, further studies are required to establish if the plasma protein binding of levofloxacin in geese is in line with that found in other avian species.

Levofloxacin was detected in all tissues selected, and the concentration was highest at 6 h and gradually decreased over 48 h. In humans approximately 90% of levofloxacin is rapidly absorbed from the intestinal tract into the hepatic portal vein and, as with other fluoroquinolones, is primarily excreted unchanged from the kidney in the urine (Fish and Chow 1997). Hence, it was reasonable to expect a higher drug residue in liver and kidney (Figure 2, Table 3). Probable tropisms related to levofloxacin have not yet been evaluated. The tissue depletion profile found in the present study was in line with that found in chickens (Kyuchukova et al. 2013; Lee et al. 2017). In this study, muscle levofloxacin concentrations, normalised for dose, were higher than concentrations found by Lee et al. (2017) and Kyuchukova et al. (2013) in chickens. These differences could be due to species specific difference, or the diverse analytical techniques used.

The MRL for many fluoroquinolones in poultry liver is about 0.1 µg/g (Anonymous 1997, 1999, 2002). On the basis of this value, a preliminary withdrawal time has been computed with the CI of 95% for liver, resulting in a time of 89.7 h. Despite the fact that this matched well with the data reported by Ravikumar et al. (2015) in chickens (4 d), caution should be taken because of the small population sample size. Further studies are required to confirm this finding.

Drug penetration in tissue can be described using the $AUC_{tissue}/AUC_{plasma}$ ratio. A ratio value over 1 indicates relatively higher drug concentrations in the tissue than in blood, with potential for tissue accumulation (Bellmann et al. 2004). The $AUC_{tissue}/AUC_{plasma}$ ratios in the current study were high in all tissues, and especially in liver (Table 3). Further studies are needed to clarify this point (*e.g.* whether levofloxacin may be stored specifically in hepatocytes).

In conclusion, a single oral dose (5 mg/kg) of levofloxacin might be effective against bacteria with a MIC <0.24 µg/ml in geese. However, further pharmacodynamic studies are needed to assess the efficacy of levofloxacin in healthy, as well as diseased, Bilgorajska geese. Liver and kidney had the highest concentrations of levofloxacin compared to other organs tested, suggesting that drug accumulation might be an issue. The authors would like to emphasise that the results of this study were purely experimental and the use of levofloxacin in avian species is not encouraged.

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In vitro and ex vivo antibacterial activity of levofloxacin against *Pasteurella multocida* and *Escherichia coli* isolated from rabbits (*Oryctolagus cuniculus*) – A preliminary study

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Abstract

Levofloxacin veterinary formulations are available in Argentina, China and India for the use in dogs, cattle, pig and sheep, but not currently in the rabbit. Only the extra-label use in rabbits is possible. Levofloxacin is not labelled for veterinary use in the EU or the USA. The activity of levofloxacin against rabbit pathogens *Pasteurella multocida* (*P. multocida*) and *Escherichia coli* (*E. coli*) was evaluated. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined in broth and serum for 10 *P. multocida* isolates and 5 *E. coli* isolates from rabbits. One isolate of each bacterial species was used for the time-killing curve study in vitro and ex vivo. In vitro AUC_{24}/MIC ratios were used for building the inhibitory pharmacodynamic I_{max} model. The *P. multocida* MIC were 0.008–0.5 μg/mL, MBC – 0.015–0.5 μg/mL. *Escherichia coli* MIC was 0.008–0.03 μg/mL and MBC – 0.03–0.25 μg/mL. Bacterial counts were reduced to the limit of detection after 24 h with levofloxacin concentrations of 2 MIC and higher. All serum samples from rabbits treated with levofloxacin eliminated the bacteria within 24 h. AUC_{24}/MIC ratios for bacteriostatic, bactericidal and bacterial elimination effects for *P. multocida* and *E. coli* isolates were 21, 29 and 75 h and 27, 32 and 60 h, respectively. Proposed daily doses against *P. multocida* (MIC = 0.015 μg/mL) and *E. coli* (MIC = 0.03 μg/mL) isolates were calculated as ≤0.91 and ≤1.43 mg/kg, respectively. Fluoroquinolones are categorized by WHO as 'highest priority critically important antimicrobials'. Considering the increasing importance of antimicrobial stewardship, antimicrobials from a lower importance category that are active against the isolate of interest should be used in preference to fluoroquinolones. Fluoroquinolone use in veterinary medicine should be based on antimicrobial susceptibility testing in order to mitigate the risk to public health and prevent the spread of bacterial resistance.

KEYWORDS
Escherichia coli, levofloxacin, *Pasteurella multocida*, rabbit, time-killing curves

1 | INTRODUCTION

Rabbits (*Oryctolagus cuniculus*) are becoming more popular as companion animals (D'Amico et al., 2022). Also, rabbits are kept as laboratory animals and food-producing animals (Toutain et al., 2010). Rabbits are prone to infectious diseases, frequently caused by Gram-negative bacteria *Pasteurella multocida* (*P. multocida*) and *Enterobacteriaceae* family, including *Escherichia coli* (*E. coli*). *Pasteurella multocida* in rabbits can cause productive rhinitis, conjunctivitis, otitis, subcutaneous abscesses, bronchopneumonia, metritis and pyometra (EFSA Panel on Animal Health and Welfare (AHAW) et al., 2021; Jekl, 2021; Percy & Barthold, 2008). *Escherichia coli* infection in rabbits is generally associated with neonatal and post-weaning colibacillosis, accompanied by gastrointestinal tract pathology (Anses, 2020; EFSA Panel on Animal Health and Welfare (AHAW) et al., 2021; El-Ashram et al., 2020).

Fluoroquinolone antimicrobials are among the most important drugs in the treatment of bacterial infections in animals (Papich, 2018). Fluoroquinolones are categorized by WHO (2019) as 'highest priority critically important antimicrobials'. Considering the increasing importance of antimicrobial stewardship principles (Lloyd & Page, 2018), antimicrobials of a lower importance category, active against the isolate of interest, should be used in preference to fluoroquinolones. Wherever possible, fluoroquinolone use in veterinary medicine should be based on antimicrobial susceptibility testing in order to mitigate the risk to public health and prevent the spread of bacterial resistance (EMA/CVMP/CHMP/682198/2017, 2020). Currently, low levels of resistance to fluoroquinolones were reported in *P. multocida* isolates (EFSA Panel on Animal Health and Welfare (AHAW) et al., 2021; Gardhouse et al., 2017; Jekl, 2021; Wang et al., 2019). *Escherichia coli* resistance to fluoroquinolones, including levofloxacin, was reported in animals (Marco-Fuertes et al., 2022; Sitovs et al., 2021). Levofloxacin is being used in both human and veterinary medicine (Sitovs et al., 2021). In some countries, such as Argentina, China and India, veterinary levofloxacin formulations are approved for dogs, cattle, pigs and poultry (Sitovs et al., 2021), but not rabbits. In the EU and the USA, levofloxacin is not currently labelled for veterinary use. More information on levofloxacin pharmacokinetics and pharmacodynamics could be useful for the effective use of this drug. Pharmacokinetic profiles of levofloxacin in rabbits were previously described (Destache et al., 2001; Sitovs et al., 2020).

In order to minimize risks and make antimicrobial therapy more effective, dosage regimen optimization is necessary (Toutain et al., 2002). The use of pharmacokinetic–pharmacodynamic integration is a proven tool for dose optimization (Toutain & Lees, 2004). The approach that is based on bacterial time-killing curves shows more rationality compared with the approach based only on minimal inhibitory concentration value, which is a static parameter (Ambrose et al., 2007).

The aims of this study were to evaluate levofloxacin's antibacterial activity against *P. multocida* and *E. coli* isolated from rabbits and to calculate proposed daily doses for parenteral (subcutaneous or intramuscular) levofloxacin administration.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates

This study included *P. multocida* clinical isolates ($n = 10$), *E. coli* isolates ($n = 5$) and commercially available *E. coli* ATCC 25922 (ATCC) as a reference strain. All *E. coli* isolates were collected from rectal swabs of clinically healthy rabbits that did not previously receive any treatment. Health status was verified by the veterinarian, based on the physical examination and complete blood analysis. Rabbits were housed on a farm near Riga, Latvia. Rectal swabs from were obtained using TRANSWAB® Gel Amies Plain (MWE) with gel media. Within the same day, the samples were transported to the laboratory of microbiology at Riga Stradins University. Swabs were cultured on McConkey agar and identified with VITEK2 Compact system (bioMérieux). One *E. coli* isolate from one rabbit was selected. Isolates were considered part of commensal flora and not pathogenic. All *P. multocida* isolates were from rabbits with clinical rhinitis and/or pneumonia. Six *P. multocida* isolates were provided by the Faculty of Veterinary Medicine, Latvia University of Life Sciences and Technologies (Jelgava, Latvia), and 4 isolates were provided by the Institute of Food Safety Animal Health and Environment BIOR (Riga, Latvia). One *P. multocida* isolate from one rabbit was used in this study.

2.2 | Determination of minimum inhibitory and minimum bactericidal concentrations in broth and serum

Minimum inhibitory concentration (MIC) values were determined using the microdilution method according to the CLSI guidelines M100 (CLSI, 2018a, 2018b). Levofloxacin standard (>99%) was purchased from Sigma-Aldrich. Levofloxacin stock solution (5120 μg/mL) was prepared in Milli-Q ultra-purified water (Millipore) with the addition of 0.1 M NaOH and further diluted to working concentrations with cation-adjusted Mueller Hinton broth (MHB) or in commercially available drug-free sterile rabbit serum (Biowest), respectively. *Escherichia coli* MIC and minimal bactericidal concentration (MBC) were detected in MHB and serum. *Pasteurella multocida* MIC and MBC were determined in MHB with the addition of 5% defibrinated sheep blood (bioTRADING Benelux B.V.) and in serum. After the overnight growth on agar plates, colonies were suspended in MHB to reach the same turbidity as the McFarland turbidity standard of 0.5.

Each *E. coli* culture was diluted 1:100 in MHB to obtain a bacterial count of approximately 10^6 colony-forming units per millilitre (CFU/mL); each *P. multocida* culture was diluted 1:100 in MHB

supplemented with 5% defibrinated sheep blood. Levofloxacin 128 μg/mL working solutions were prepared in MHB and in serum. Final incubation for 24 h at 37°C was performed with levofloxacin serial dilutions from 64 to 0.004 μg/mL in both media in the presence of 5×10^5 CFU/mL of bacteria. After the incubation, *E. coli*-containing microdilution plates were read at 600 nm using Infinite F50 Plus reader (Tecan). MIC was reported as the lowest levofloxacin concentration, which showed no turbidity in the microdilution tray wells. For *P. multocida* in MHB with blood, MIC was reported as the lowest concentration where no colour change from red to brown was visually observed.

To determine the MBC, 10 μL of the content of wells showing no bacterial growth was transferred to plates, containing Tryptic Soy Agar (TSA) for *E. coli* and TSA supplemented with 5% defibrinated sheep blood for *P. multocida*. After incubation for 24 h at 37°C, colonies were counted. The limit of detection was 100 CFU/mL. The lowest concentration showing no bacterial growth was reported as MBC. Reference culture *E. coli* ATCC 25922 MIC and MBC values were determined on MHB only. Experiments were performed in triplicate.

2.3 | Levofloxacin serum samples for ex vivo bacterial killing curve evaluation

Serum samples containing levofloxacin at known concentrations were obtained from the study of Sitovs et al. (2020). The experimental protocol was approved by the Animal Ethics Committee of the Republic of Latvia Food and Veterinary Service (Permission 025564). In that study, a 5 mg/kg single dose of levofloxacin was administered to clinically healthy domestic rabbits, intramuscularly (IM) and subcutaneously (SC). After each drug administration, serum samples for ex vivo study were obtained after 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 h. Pooled serum samples from experimental rabbits (3 mL) were used for the present study. Levofloxacin concentrations in pooled serum samples were determined prior to the time-killing study with a validated HPLC method (Sitovs et al., 2020).

2.4 | In vitro bacterial killing curves for *Pasteurella multocida* and *Escherichia coli*

One isolate of *P. multocida* and one isolate of *E. coli* were chosen to be used in the bacterial time-killing curve study. The bacterial killing curve study protocol was based on the method described by Lee et al. (2017). Levofloxacin solutions in drug-free rabbit serum were prepared at concentrations relative to the MIC in the serum of the bacterial isolate. For *P. multocida*, concentrations were 0.00 μg/mL (control), 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 times the MIC and for *E. coli* concentrations were 0.00 μg/mL (control), 0.5, 1, 2, 4, 8, 16 and 32 times the MIC. For each bacterial isolate, 8 colonies from overnight growth on agar plates were added to 9 mL of MHB and incubated for 20 h at 37°C in presence of 5% CO_2. Ten microlitres of broth culture were added to 1 mL of levofloxacin solutions in serum in order to reach the concentration of approximately 1.6×10^6 CFU/mL for *P. multocida* isolate and 2×10^7 CFU/mL *E. coli*. Samples were incubated for 24 h at 37°C in an orbital shaker; 20 μL from all samples were withdrawn at 3, 6 and 24 h of incubation. Prior to withdrawal, samples were vortexed. Dilutions ranging from 10^{-1} to 10^{-8} in sterile 0.9% saline were prepared to count the CFU. A 10 μL volume of each saline dilution was inoculated on a TSA plate and incubated for 16 h. TSA plates for *P. multocida* samples were supplemented with 5% defibrinated sheep blood. CFU were counted and the limit of detection was 100 CFU/mL. The count of bacteria in the initial inoculum was approved with the same dilution in the sterile saline method. All experiments were performed in triplicate.

2.5 | Ex vivo bacterial killing curves for *Escherichia coli* and *Pasteurella multocida*

The same *P. multocida* and *E. coli* isolates, as for the in vitro bacterial killing study, were used in the ex vivo study. The study protocol was almost identical, to the in vitro bacterial killing. The difference was that instead of levofloxacin dilutions in antibiotic-free rabbit serum, we used serum samples obtained from rabbits that received 5 mg/kg of levofloxacin parenterally. Pooled serum samples collected at 0, 0.5, 1, 2, 4, 8 and 10 h after administration contained 0.00, 3.26, 2.64, 1.48, 0.58, 0.13 and 0.07 μg/mL for IM and 0.00, 2.59, 2.70, 1.91, 0.75, 0.14 and 0.08 μg/mL for SC routes of administration, respectively. Levofloxacin concentrations were determined immediately prior to this study with a validated HPLC method described by Sitovs et al. (2020). All experiments were performed in triplicate.

2.6 | Pharmacodynamic modelling and daily dose calculation

To determine AUC_{24}/MIC ratios, each in vitro levofloxacin concentration was multiplied by 24 (period of incubation) and then divided by the MIC value of each bacterial isolate tested, respectively. The relationship between in vitro AUC_{24}/MIC and $\log_{10}$ difference in bacterial count from the initial inoculum to the bacterial count after 24 h of incubation for serum was evaluated by using the sigmoid inhibitory I_{max} model in Phoenix WinNonlin (Certara). Akaike's Information Criterion was applied to determine the goodness of fit. The model is described with the following equation:

$$E = E_0 - \frac{I_{max} \times C^{\gamma}}{C^{\gamma} + IC_{50}^{\gamma}}$$

E – antibacterial effect of levofloxacin; I_{max} – difference between $\log_{10}$ difference in bacterial count between 0 and 24 h in the control sample ($\log E_0$) and the $\log_{10}$ difference in bacterial count in the sample incubated with levofloxacin for 24 h when the limit of detection of 100 CFU/mL is reached; E_0–$\log_{10}$ difference in the bacterial count

from 0 to 24 h of incubation in the control sample, antibiotic-free; IC_{50} is the AUC_{24}/MIC producing 50% of the maximal antibacterial effect; C is the AUC_{24}/MIC in the effect compartment (serum); γ– the Hill coefficient which characterizes the slope of the AUC_{24}/MIC response curve.

The antibacterial activity of levofloxacin against both bacteria species in this study was assessed by calculation of AUC_{24}/MIC values required for bacteriostatic, bactericidal effects and bacterial elimination. AUC_{24}/MIC for bacteriostatic effect was calculated using $E=0$, that is, no change in bacterial counts after the incubation for 24 h with levofloxacin. AUC_{24}/MIC for bactericidal effect was calculated using $E=-3$, that is, bacterial counts reduction by 99.9% after the incubation for 24 h with levofloxacin. AUC_{24}/MIC for bacterial elimination effect was calculated using the lowest E value when the maximal antibacterial effect was reached, that is, bacterial count reduction to the limit of quantification (100 CFU/mL) after the incubation for 24 h with levofloxacin.

Obtained from pharmacokinetic–pharmacodynamic integration, antibacterial effects AUC_{24}/MIC values were used to calculate optimal doses for three effect levels – bacteriostatic, bactericidal and bacterial elimination. The following formula (McKellar et al., 2004) was used:

$$\text{Dose per day} = \frac{\frac{\text{AUC24}}{\text{MIC}} \times \text{MIC} \times \text{Cl}}{f_u \times F \times 24}$$

where AUC_{24}/MIC are ratios for bacteriostatic, bactericidal and bacterial elimination effects, MIC is minimum inhibitory concentration in serum, Cl is clearance, F is bioavailability, and f_u is a free fraction of levofloxacin in plasma. As reported by Sitovs et al. (2020), the following values were used, Cl = 0.6 mL/g/h and $F=1$. As reported by Destache et al. (2001), levofloxacin protein binding in rabbit plasma was 25%, thus, $f_u=0.75$.

3 | RESULTS

3.1 | Minimal inhibitory and minimal bactericidal concentration

All 10 isolates of *P. multocida* and all six isolates (including reference strain) of *E. coli* were susceptible to levofloxacin. None of the isolates were considered resistant. MIC and MBC values and MBC/MIC ratios in both media of all bacterial isolates are represented in Tables 1 and 2. Year of isolate collection is provided in Table 1, as well as diagnosis and origin of isolate.

3.2 | In vitro antibacterial activity of levofloxacin and time-killing curves

Figure 1 represents the time-dependent antibacterial activity of levofloxacin in vitro against a selected isolate of *P. multocida* (Isolate Nr. 7697, MIC = 0.015 μg/mL). In the absence of the drug, the 24-h incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.25 and 0.5 MIC were not able to inhibit bacterial growth, and after 24 h of incubation, bacterial counts exceeded the initial inoculum. One MIC concentration reduced the bacterial growth, but after 24 h of incubation, the bacterial count was similar to the initial inoculum. Concentrations of levofloxacin equal to 2 and 4 MIC reduced the number of bacteria gradually at 3 and 6 h of incubation and eradicated the bacteria at 24 h of incubation. Levofloxacin concentrations higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 h of incubation.

Figure 2 shows the time-dependent antibacterial activity of levofloxacin in vitro against a selected isolate of *E. coli* (Isolate Nr. 1, MIC = 0.03 μg/mL). In the absence of the drug, the 24-h incubation resulted in bacterial growth of approximately 3 $\log_{10}$ CFU/mL. Levofloxacin concentrations equivalent to 0.5 and 1 MIC were not able to inhibit bacterial growth, and after 24 h of incubation, bacterial counts exceeded the initial inoculum. Concentrations of levofloxacin equal to 2 MIC reduced the number of bacteria gradually at 3 and 6 h of incubation and eliminated the bacteria after 24 h of incubation. Levofloxacin concentrations equal to and higher than 4 MIC decreased the number of bacteria to the limit of detection already at 3 h of incubation.

3.3 | Ex vivo antibacterial activity of levofloxacin after intramuscular and subcutaneous administration and time-killing curves

Figures 3 and 4 represent the bacterial time-killing curves for levofloxacin ex vivo against a selected isolate of *P. multocida* (isolate Nr. 7697, MIC = 0.015 μg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits. Concentrations of levofloxacin achieved in serum after 0.5, 1, 2 and 4 h of both IM and SC administration reduced the bacterial count to the limit of detection already after 3 h of incubation. Considering 25% protein binding, free levofloxacin concentrations in these serum samples were 2.45 (163 MIC), 1.98 (132 MIC), 1.11(74 MIC) and 0.44 (29 MIC) μg/mL, and 1.94 (130 MIC), 2.03 (135 MIC), 1.43 (96 MIC) and 0.56 (38 MIC) μg/mL for IM and SC samples, respectively. After incubation for 24 h, all serum samples containing levofloxacin were able to reduce the *P. multocida* bacterial count to the limit of quantification.

Figures 5 and 6 represent the bacterial time-killing curves for levofloxacin ex vivo against a selected isolate of *E. coli* (isolate Nr. 1, MIC = 0.03 μg/mL) after IM and SC dosage of 5 mg/kg body weight of levofloxacin solution to rabbits. Only serum samples collected at 0.5, 1 and 2 h, representing the highest drug concentrations, were able to reduce the bacterial count to the limit of quantification after 3 h of incubation. Considering 25% protein binding, free levofloxacin concentrations in these serum samples were 2.45 (82 MIC), 1.98 (66 MIC) and 1.11(37 MIC) μg/mL, and 1.94 (65 MIC), 2.03 (68 MIC) and 1.43 (48 MIC) μg/mL for IM and SC samples, respectively. After

TABLE 1 Minimal inhibitory concentration and minimal bactericidal concentration of *Pasteurella multocida* isolates from rabbits.

	MIC_{broth} (μg/mL)	MIC_{serum} (μg/mL)	MBC_{broth} (μg/mL)	MBC_{serum} (μg/mL)	MBC/MIC_{broth}	MBC/MIC_{serum}	Diagnosis and isolate origin
P. multocida 297 (2021)	0.03	0.03	0.06	0.125	2	4	Nasal catarrh, pneumonia Nasal swab
P. multocida 320 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 306 (2021)	0.03	0.03	0.125	0.125	4	4	Rhinitis, Nasolacrimal flush fluid
P. multocida 122 (2021)	0.008	0.008	0.008	0.015	1	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 2101 (2021)	0.008	0.008	0.015	0.015	2	2	Rhinitis, Nasal swab
P. multocida 298 (2021)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasolacrimal flush fluid
P. multocida 769[a] (2022)	0.015	0.015	0.03	0.03	2	2	Rhinitis, Nasal swab
P. multocida 3178 (2022)	0.008	0.008	0.125	0.125	16	16	Rhinitis, Nasolacrimal flush fluid
P. multocida 7042 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid
P. multocida 0634 (2022)	0.5	0.5	0.5	0.5	1	1	Rhinitis, Nasolacrimal flush fluid

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.

[a]*P. multocida* isolate selected for in vitro and ex vivo bacterial time-killing study.

TABLE 2 Minimal inhibitory concentration and minimal bactericidal concentration of *Escherichia coli* reference strain ATCC25922 and isolates from rabbits.

	MIC_{broth} (μg/mL)	MIC_{serum} (μg/mL)	MBC_{broth} (μg/mL)	MBC_{serum} (μg/mL)	MBC/MIC_{broth}	MBC/MIC_{serum}
E. coli ATCC 25922	0.03	-	0.03	-	1	-
E. coli 1[a]	0.03	0.03	0.25	0.25	8	8
E. coli 2	0.008	0.008	0.03	0.03	4	4
E. coli 5	0.015	0.015	0.06	0.06	4	4
E. coli 11	0.015	0.015	0.03	0.06	2	4
E. coli 12	0.008	0.008	0.03	0.03	4	4

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration.
[a]*E. coli* isolate selected for in vitro and ex vivo bacterial time-kill study.

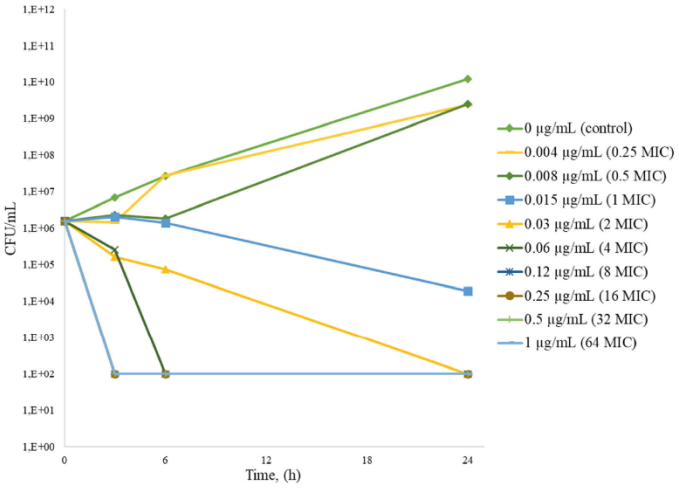

FIGURE 1 In vitro time-killing curves representing the growth of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 μg/mL) with different levofloxacin concentrations in rabbit serum. Standard error bars are excluded for clarity.

incubation for 24 h, all serum samples containing levofloxacin were able to reduce the *E. coli* bacterial count to the limit of quantification.

3.4 | Pharmacodynamic modelling and daily dose calculation

For the pharmacodynamic analysis, the plots of AUC_{24}/MIC ratios versus changes in bacterial counts after 24 h of incubation for selected *P. multocida* and *E. coli* isolates are presented in Figures 7 and 8, respectively. Pharmacodynamic data obtained from the I_{max} model, namely, AUC_{24}/MIC required for bacteriostatic, bactericidal and bacterial elimination for selected *P. multocida* and *E. coli* isolates, are presented in Tables 3 and 4, respectively. Calculated daily doses of parenteral levofloxacin required to achieve antibacterial effects are reported in Table 5. Calculated daily doses for *P. multocida* isolates exhibiting highest MIC value (0.5 μg/mL) are 8.30, 11.55 and 30.18 mg/kg daily, for bacteriostatic, bactericidal and bacterial elimination effects, respectively.

4 | DISCUSSION

To the best of the authors' knowledge, this study describes for the first time levofloxacin time-killing curves for *P. multocida* and *E. coli* isolates from rabbits.

None of the bacterial isolates included in this study showed resistance to levofloxacin. However, reports are indicating cases of *P. mulocida* and *E. coli* resistance to this drug (Saha et al., 2021; Sitovs et al., 2021). MIC values for both *P. mulocida* and *E. coli* were low, compared to other pathogens' MIC reported in the literature (Sitovs et al., 2021). Two *P. multocida* isolates (Nr. 7042 and 0634) showed relatively high MIC (0.5 μg/mL). As no clinical breakpoints for levofloxacin for *P. multocida* isolates from rabbits currently exist, applying CLSI M100 (2018a) levofloxacin breakpoints, these isolates could be considered susceptible. Applying fluoroquinolone clinical breakpoints for respiratory *P. multocida* (pradofloxacin, enrofloxacin and danofloxacin) according to the CLSI VET08 (2018b), these isolates would not be considered susceptible, anymore (susceptible defined as MIC ≤ 0.25 μg/mL), but rather intermediate. All other *P. multocida*

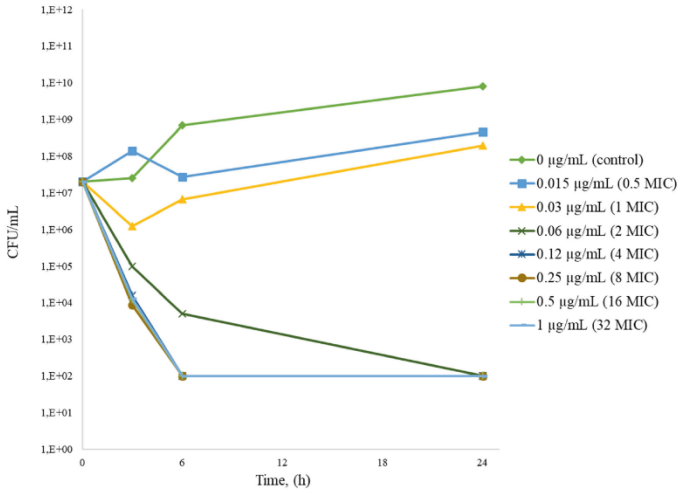

FIGURE 2 In vitro time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in rabbit serum. Standard error bars are excluded for clarity.

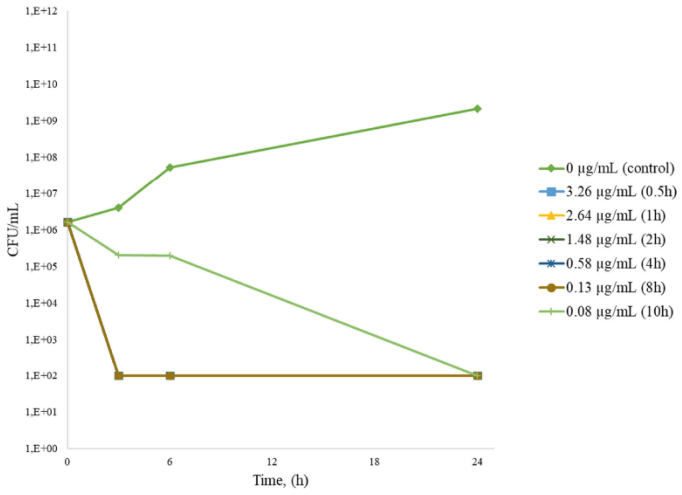

FIGURE 3 Ex vivo time-killing curves representing the growth of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 µg/mL) with different levofloxacin concentrations in serum samples obtained after intramuscular dose of 5 mg/kg to healthy rabbits ($n = 6$). Standard error bars are excluded for clarity.

isolates showed MIC values (0.008–0.03 µg/mL) in line with MIC_{90} values reported for veterinary fluoroquinolones and their active metabolites – difloxacin, enrofloxacin, ciprofloxacin, marbofloxacin, orbifloxacin and pradofloxacin (0.008–0.05 µg/mL) against *P. multocida* (Papich, 2018). MIC_{90} values for the same veterinary fluoroquinolones against *E. coli* (0.03–0.39 µg/mL) were slightly higher compared to *E. coli* MIC values obtained in the present study (0.008–0.03 µg/mL). Only 15 bacterial isolates were used in our study; thus, it is not yet obvious that levofloxacin is significantly superior to other veterinary fluoroquinolones.

Minimal bactericidal concentration/MIC ratios of levofloxacin were not high in the present study. The median ratios for *P. multocida* and *E. coli* isolates were 2 and 4, respectively. That is similar to ratios obtained from isolates from humans by Akinjogunla et al. (2022). In that study, levofloxacin was reported to achieve a reduction in CFU/mL of ≥99.9% of most aetiology of bacteremia faster compared to other fluoroquinolones. MBC/MIC ratios >8 were reported to be associated with antibiotic tolerance (Gonzalez et al., 2013). Our pharmacodynamic study results do not suggest levofloxacin tolerance in rabbits.

Aliabadi and Lees (2001) describe AUC_{24}/MIC as the most important factor to determine efficacy of concentration-dependent antibacterial drugs, including fluoroquinolones. In the present study, the use of ex vivo AUC_{24}/MIC was not suitable for pharmacokinetic–pharmacodynamic modelling. The reason for that was bacterial count reduction to the detection limit after

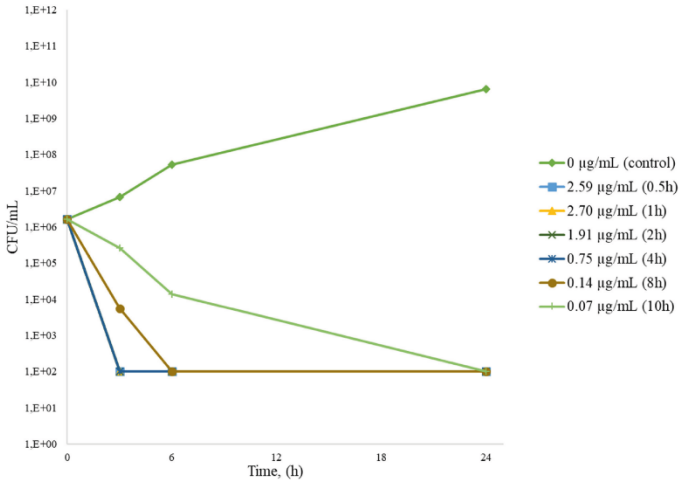

FIGURE 4 Ex vivo time-killing curves representing the growth of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous dose of 5 mg/kg to healthy rabbits ($n = 4$). Standard error bars are excluded for clarity.

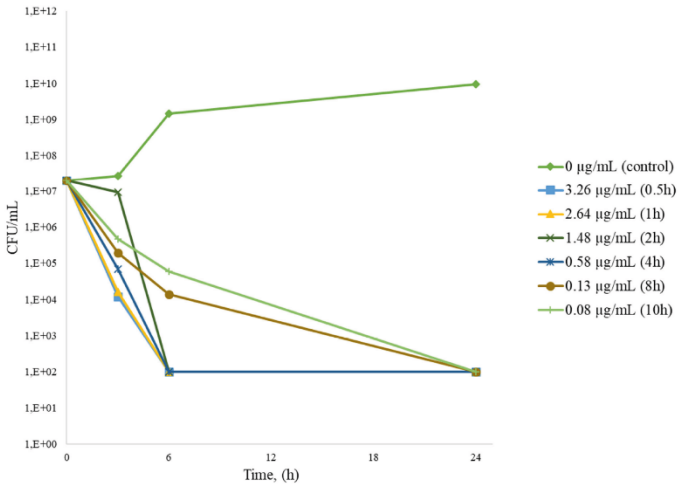

FIGURE 5 Ex vivo time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in samples obtained after intramuscular dose of 5 mg/kg to healthy rabbits ($n = 6$). Standard error bars are excluded for clarity.

24 h of incubation with all experimentally obtained levofloxacin concentrations in rabbit serum. All samples from time points collected after IM and SC dose of 5 mg/kg had levofloxacin concentrations higher than 1 MIC for both bacterial isolates used in the time-killing study. In vitro AUC_{24}/MIC data were used for modelling instead. AUC_{24}/MIC values obtained for lower levofloxacin concentrations (0.25, 0.5 and 1 MIC, which did not reduce the bacterial counts to the detection limit) provided more data for creating the model. When time-killing curves for in vitro and ex vivo experiments were visually compared, their similarity provided almost identical bacterial killing patterns. That justifies the use of in vitro AUC_{24}/MIC data for modelling. Slightly slower killing rate was observed in the in vitro study compared to the ex vivo study. That could be attributable to chemical differences between experimental rabbit serum and commercially available rabbit serum used for the in vitro study. Hill coefficient values in both models in this study were high, 5.64 for *P. multocida* and 9.98 for *E. coli*, respectively. These values illustrate the rapid increase in levofloxacin activity with the small increase in the concentration. Lee et al. (2017) reported a slightly less steep slope of 5.21 for levofloxacin against *E. coli* isolated from broiler chickens.

Levofloxacin in this study showed similar AUC_{24}/MIC ratios required for bacteriostatic, bactericidal and bacterial elimination effects for *P. multocida* (20.76, 28.88 and 75.46 h), compared to marbofloxacin, reported by Dorey et al. (2017) (20.9, 45.2 and 71.7 h) for *P. multocida* isolates from pigs and slightly lower than marbofloxacin

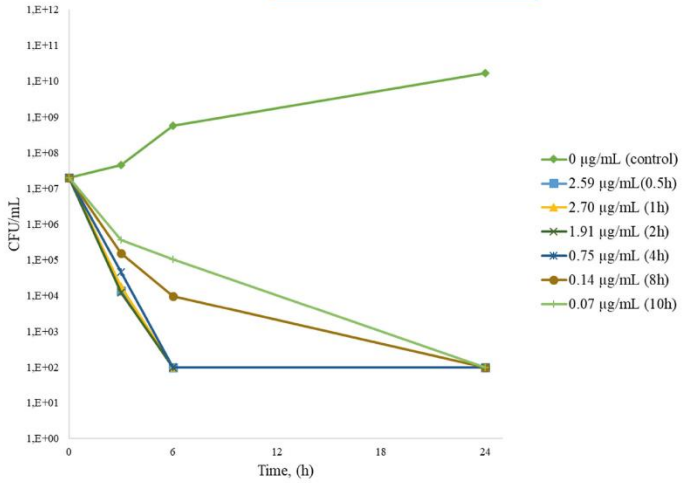

FIGURE 6 Ex vivo time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous dose of 5 mg/kg to healthy rabbits ($n = 4$). Standard error bars are excluded for clarity.

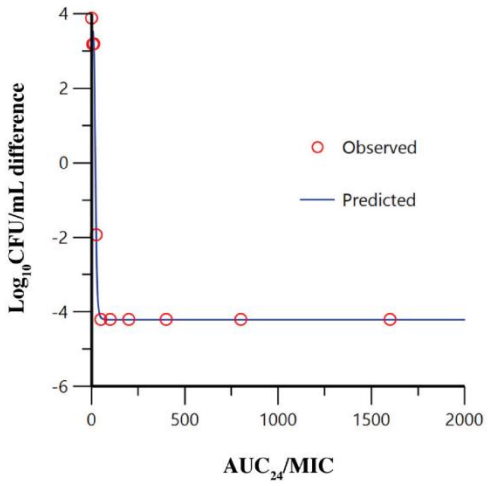

FIGURE 7 Plot of in vitro AUC_{24}/MIC versus *Pasteurella multocida* (Nr. 7697, MIC = 0.015 µg/mL) bacterial count difference in levofloxacin containing rabbit serum.

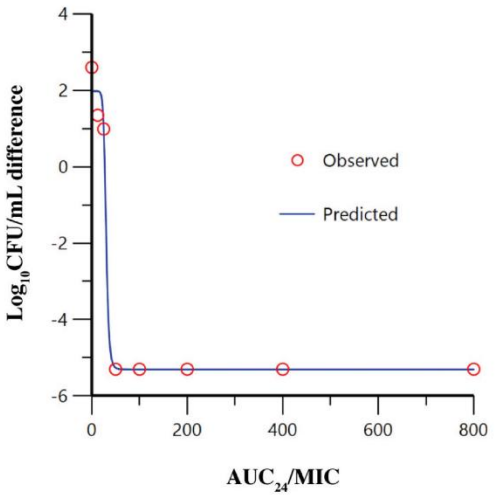

FIGURE 8 Plot of in vitro AUC_{24}/MIC versus *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) bacterial count difference in levofloxacin containing rabbit serum.

reported by Potter et al. (2013) for isolates from calves (48.6, 64.9 and 74.8 h, respectively).

AUC_{24}/MIC ratios for bacteriostatic, bactericidal and bacterial elimination effects in this study for *E. coli* (27.25, 32.49 and 59.62 h) were higher compared to values reported by Lee et al. (2017) – 18.77, 24.02 and 36.27 h, respectively. AUC_{24}/MIC ratios obtained by Haritova et al. (2006) for danofloxacin against *E. coli* isolated from turkeys were significantly lower (0.42, 1.90 and 6.73 h) and for enrofloxacin against *E. coli* isolated from chickens were much higher (257.40 and 2794.40 h for bacteriostatic effect and bacterial elimination, respectively; Haritova & Russenova, 2010).

Despite the previous conclusion that a dose of 5 mg/kg levofloxacin is unlikely to be effective in rabbits (Sitovs et al., 2020), the ex vivo time-killing curves showed a reduction of the bacterial counts to the limit of quantification at 24 h. Calculated daily doses appear to be even lower. In this study, proposed doses per day required for bacteriostatic, bactericidal and bacterial elimination effects (0.25–1.43 mg/kg daily) were lower compared to

TABLE 3 Pharmacokinetic–pharmacodynamic levofloxacin data integration of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 μg/mL) in vitro growth inhibition.

Parameter	Units	Estimated value
I_{max}	CFU/mL	7.75
E_0	CFU/mL	3.54
E_0–I_{max}	CFU/mL	−4.21
IC_{50}	h	21.41
AUC_{24}/MIC Bacteriostatic	h	20.76
AUC_{24}/MIC Bactericidal	h	28.88
AUC_{24}/MIC Bacterial elimination	h	75.46
Slope (γ)	N/A	5.64

Note: I_{max}—difference between $\log_{10}$ difference in bacterial count between 0 and 24 h in the control sample ($\log E_0$) and the $\log_{10}$ difference in bacterial count in the sample incubated with levofloxacin for 24 h when the limit of detection of 100 CFU/mL is reached.

E_0—$\log_{10}$ difference in the bacterial count from 0 to 24 h of incubation in the control sample.

E_0–I_{max}—$\log_{10}$ difference in the bacterial count from 0 to 24 h of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached.

IC_{50}—AUC_{24}/MIC producing 50% of the maximal antibacterial effect.

γ—the Hill coefficient, slope of the AUC_{24}/MIC response curve.

Abbreviation: N/A, not applicable.

TABLE 4 Pharmacokinetic–pharmacodynamic levofloxacin data integration of *Escherichia coli* (Nr. 1, MIC = 0.03 μg/mL) in vitro growth inhibition.

Parameter	Units	Estimated value
I_{max}	CFU/mL	7.28
E_0	CFU/mL	1.98
E_0–I_{max}	CFU/mL	−5.30
IC_{50}	h	30.08
AUC_{24}/MIC Bacteriostatic	h	27.25
AUC_{24}/MIC Bactericidal	h	32.49
AUC_{24}/MIC Bacterial elimination	h	59.62
Slope (γ)	N/A	9.98

Note: I_{max}—difference between $\log_{10}$ difference in bacterial count between 0 and 24 h in the control sample ($\log E_0$) and the $\log_{10}$ difference in bacterial count in the sample incubated with levofloxacin for 24 h when the limit of detection of 100 CFU/mL is reached.

E_0—$\log_{10}$ difference in the bacterial count from 0 to 24 h of incubation in the control sample.

E_0–I_{max}—$\log_{10}$ difference in the bacterial count from 0 to 24 h of incubation in samples incubated with levofloxacin when the detection limit of 100 CFU/mL is reached.

IC_{50}—AUC_{24}/MIC producing 50% of the maximal antibacterial effect.

γ—the Hill coefficient, slope of the AUC_{24}/MIC response curve.

Abbreviation: N/A, not applicable.

TABLE 5 Calculated daily doses of levofloxacin for parenteral administration to rabbits against *Pasteurella multocida* (MIC = 0.015 μg/mL) and *Escherichia coli* (MIC = 0.03 μg/mL).

Dose per day	*P. multocida* (MIC = 0.015 μg/mL), mg/kg	*E. coli* (MIC = 0.03 μg/mL), mg/kg
Bacteriostatic effect	0.25	0.65
Bactericidal effect	0.35	0.78
Bacterial elimination	0.91	1.43

the levofloxacin doses calculated for broilers by Lee et al. (2017) (1.1–4.3 mg/kg daily) and for rabbits – 29 mg/kg daily (Sitovs et al., 2020). Previously reported dose was up to 100-fold higher that doses obtained in this study. Compared to the dose reported by Sitovs et al. (2020), current study utilizes experimental pharmacodynamic data from susceptible bacterial time-killing curves, while previous pharmacodynamic data were from the published literature. Difference in doses between two studies originates from the higher AUC_{24}/MIC used in calculations – 72 h, as reported by Madsen et al. (2019) and with lower MIC values used in calculations. In the current study, doses were calculated based on the experimentally obtained MIC values, while Sitovs et al. (2020) used MIC = 0.5 μg/mL. Doses calculated using highest *P. multocida* MIC (0.5 μg/mL) are less different from the dose reported by Sitovs et al. (2020), 8.30, 11.55 and 30.18 vs. 29 mg/kg daily. Real, rather than theoretical MIC values were used in dose calculations here. Sitovs et al. (2020) also reported that levofloxacin bioavailability in rabbits after IM and SC routes of administration is around 100%; thus, complete bioavailability is expected. From the point of view of bioavailability, there is no difference between IM and SC administration for suggested daily doses. However, compared to SC, the IM administration is generally more painful and considering relatively small muscle mass in rabbits, rarely used (Shellim, 2011).

Additional factors that can contribute to the calculation of daily doses are associated with changes in fluoroquinolone pharmacokinetics in rabbits in the diseased state. Abo-el-Sooud and Goudah (2010) reported that *P. multocida* infection resulted in a change in the primary pharmacokinetic parameter clearance for marbofloxacin. If the same could apply to levofloxacin, that may impact the calculation of the dose. To prove this, an additional pharmacokinetic study of levofloxacin in infected animals would be required.

The authors are aware of the limitations of this study. First, a small number of animals in the pharmacokinetic study do not cover all possible inter-animal difference in clearance, necessary for dose calculation. Impact of infection was not considered in this study, as serum samples from healthy rabbits were used. Small number of bacterial isolates used in this study does not represent all MIC variability within one isolate and among population of wild-type pathogenic bacteria in rabbits. The ex vivo study does not take into account the immune response of the animal organism, which could contribute to the elimination of bacteria and possibly allow lower doses of the antimicrobial agent to be used. The effect of inoculum

concentration was not assessed in terms of antimicrobial activity of levofloxacin. Finally, this study did not predict further resistance development against levofloxacin for the tested microbial isolates, and no mutant prevention concentrations values were obtained in this study. However, fluoroquinolone resistance is an important issue in global health (Brown, 1996; WHO, 2019). Lastly, consideration of antimicrobial stewardship principles (Lloyd & Page, 2018) in the selection and possible use of levofloxacin in rabbits has to be considered.

5 | CONCLUSION

Our study has shown that levofloxacin is active against susceptible bacteria isolated from rabbits. The maximal residue limits for levofloxacin are not currently defined. That restricts levofloxacin use in food-producing animals. Our current study provides a preliminary examination of key elements of the dose regimen in companion rabbits. In order to justify the use of parenteral levofloxacin in treatment of rabbit infections are needed additional both pharmacokinetic and pharmacodynamic studies.

AUTHOR CONTRIBUTIONS

AS, DB and SP conceptualized this study. AS, SP and IS contributed to sample collection and carried out the experiments. AS and DB carried out the data analysis. AS wrote the original manuscript draft and all authors contributed to and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request from the corresponding author.

ETHICS STATEMENT AND ANIMAL WELFARE

The study was approved by the Animal Ethics Committee of the Republic of Latvia Food and Veterinary Service (Permission 025564) and conducted in compliance with European law (Directive 2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

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Annex 5

Tables A1–A7

Table A1

Veterinary formulations containing levofloxacin

Country	Name	Active ingredient(s)	Dosage form	Species	Indication	Dosing	Withdrawal time
Argentina	Floxaday	Levofloxacin	Tablets	Dog	Soft tissue/ respiratory/prostate/mammary gland infections, UTI, osteomyelitis, septicaemia, pyoderma	PO 10 mg/kg every 24h	N/A
Argentina	Floxaday	Levofloxacin	Injectable solution	Dog		1.5 mL /10 kg every 24h	N/A
China	ZDHF-Levofloxacin W.S. P	Levofloxacin	Powder	Fowl	Increase poultry laying rate	100g of powder + 150kg water, twice daily	N/A
India	LEVOVET	Levofloxacin	Powder	N/A	N/A	N/A	N/A
India	Veterinary Levofloxacin Injection	Levofloxacin	Injectable solution	N/A	N/A	N/A	N/A
India	Levosept	Levofloxacin, colistin	Oral liquid	Poultry	N/A	N/A	N/A
India	LCB-Vet	Levofloxacin, colistin and bromhexine	Oral liquid	N/A	N/A	4-8 mL per 10 L of drinking water	Meat: 28 days; Eggs: 7days
Russia	Лексофлон (Lexoflon)	Levofloxacin	Injectable solution	Cattle, pig	N/A	IM injection 1 mL per 30 kg BW	Cattle/Pig (meat): 9 days; Milk: 4 days
Russia	Лексофлон OR (Lexoflon OR)	Levofloxacin	Oral liquid	Poultry, pig	N/A	1 mL per 20 kg BW (0.5 mL per 1 L drinking water)	Poultry (meat): 7 days; Pigs (meat): 9 days

BW – body weight, N/A – data not available in the reference source

Table A2

Susceptibility of Gram-negative microorganisms isolated from animals to levofloxacin

BP – biofilm producing, NBP – non-biofilm producing, ESBL – extended-spectrum beta-lactamases, MDR – multidrug resistant, STEC – Shiga toxin producing *E. coli*, EPEC – Enteropathogenic *E.coli*, APEC – avian pathogenic *E. coli*, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Acinetobacter spp.	Cattle	Healthy	176	100.0		0.0	N/A	Gurung et al., 2013
Acinetobacter baumannii	Cattle	Healthy	57	100.0		0.0	N/A	Gurung et al., 2013
Acinetobacter baumannii	Chicken	Healthy	80			37.5	N/A	Kanaan et al., 2020
Acinetobacter baumannii	Turkey	Healthy	120			37.5	N/A	Kanaan et al., 2020
Aeromonas hydrophilia	Tilapia	Diseased	1	100.0			N/A	Pauzi et al., 2020
Aeromonas hydrophilia	Rainbow trout	N/A	12	100.0			N/A	Stratev et al., 2013
Brucella abortus	Cattle	Diseased	3	100.0			N/A	Morales-Estrada et al., 2016
Brucella melitensis	Cattle	Diseased	3	66.0		33.0	N/A	Morales-Estrada et al., 2016
Brucella suis	Cattle	Diseased	1	100.0			N/A	Morales-Estrada et al., 2016
Brucella abortus	Goat	Diseased	3	100.0			N/A	Morales-Estrada et al., 2016
Bordetella hinzii	Turkey	Diseased	1	100.0			N/A	Beach et al., 2012
Bordetella avium	Turkey	Diseased	12		8.3		N/A	Beach et al., 2012
Bordetella avium	Saw-whet owl	Healthy	1	100.0			N/A	Beach et al., 2012
Citrobacter freundii	Green turtle	Diseased	1	100.0			N/A	Goldberg et al., 2019
Escherichia coli	Various (pig, chicken, duck)	Diseased	495			70.5	0.0625 - >256	Liu et al., 2012
Escherichia coli	Dog	Diseased	38			18.4	N/A	Inoue et al., 2012
Escherichia coli (ESBL 34%)	Rat	N/A	32			56.3	N/A	Onanga et al., 2020
Escherichia coli	Rabbit	Healthy	5	100.0			0.0008 - 0.03	Sitovs et al., 2023
Escherichia coli	Pig	N/A	479			38.8	N/A	Cheng et al., 2020
Escherichia coli (ESBL)	Cattle	Diseased	30			74.7	N/A	Prajapati et al., 2020
Escherichia coli (MDR)	Cattle	N/A	12			100.0	N/A	Anes et al., 2020
Escherichia coli (ESBL)	Cattle	Healthy	22	83.3	16.7	0.0	N/A	Batabyal et al., 2018
Escherichia coli	Cattle	Diseased	31	87.1			N/A	Boyal et al., 2018
Escherichia coli	Cattle	Healthy	2	S			N/A	Tanzin et al., 2016
Escherichia coli	Buffalo	Healthy	1	S			N/A	Tanzin et al., 2016
Escherichia coli	Buffalo	Diseased	15	66.6			N/A	Bhadaniya et al., 2019

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Escherichia coli	Cattle	Diseased	30	96.7	3.3	0.0	N/A	Mohanty et al., 2013
Escherichia coli (MDR)	Cattle	Healthy	500	4.6			N/A	Ajayi et al., 2011
Escherichia coli (31 STEC and 6 EPEC)	Yak	N/A	37	100.0			N/A	Bandyopadhyay et al., 2012
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	91			13.2	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	95			22.1	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	112			40.2	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	112			53.6	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	130			54.6	N/A	Chen et al., 2014
Escherichia coli	Poultry (Broiler, laying hen)	Diseased	540			38.7	N/A	Chen et al., 2014
Escherichia coli	Pig	Healthy, diseased	203			50.2	N/A	Jiang et al., 2011
Escherichia coli	Poultry (Chicken, geese, duck, partridge)	Healthy, diseased	389			20.8	N/A	Jiang et al., 2011
Escherichia coli	Pig, poultry	Healthy	300			14.0	N/A	Jiang et al., 2011
Escherichia coli	Pig, poultry	Diseased	292			48.3	N/A	Jiang et al., 2011
Escherichia coli	Duck	Healthy	10			0.0	N/A	Jiang et al., 2011
Escherichia coli	Chicken (Broiler breeder)	Healthy	37			89.1	N/A	Benameur et al., 2019
Escherichia coli	Chicken	Diseased	34	38.2	35.3	26.5	N/A	Ibrahim et al., 2019
Escherichia coli (90% APEC)	Chicken (Broiler)	Diseased (suspected)	50			50.0	N/A	Subedi et al., 2018
Escherichia coli	Chicken (Broiler)	Healthy	54			22.0	N/A	Mahmud et al., 2018
Escherichia coli (APEC)	Chicken (Broiler)	Diseased	56	98.0		2.0	0.25 - 8	Zhao et al., 2005
Escherichia coli	Duck	Diseased	25	S			N/A	Panda at al., 2010
Escherichia coli	Chicken, duck	Diseased	60	40.0	15.0	45.0	N/A	Hashem et al., 2022
Escherichia coli	Chicken (Broiler)	Healthy	150			33.0	N/A	Hussein et al., 2022
Escherichia coli	Chicken (Broiler)	Diseased	162	36.0	20.0	44.0	N/A	Jaseem et al., 2023
Escherichia coli	Pigeon	Healthy	21	100.0		0.0	N/A	Karim et al., 2020

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Enterobacter hormaechei (ESBL)	Green turtle	Diseased	1	100.0			N/A	Goldberg et al., 2019
Francisella tularensis subsp. *holarctica*	Various (hare, vole)	N/A	32	S			< 0.25	del Blanco et al., 2004
Fusobacterium spp.	Buffalo	Diseased	5	100.0			N/A	Bhadaniya et al., 2019
Haemophilus parasuis (BP)	Pig	Diseased	73			24.7	N/A	Zhang et al., 2014
Haemophilus parasuis (NBP)	Pig	Diseased	37			24.3	N/A	Zhang et al., 2014
Haemophilus parasuis	Pig	Diseased	143			20.3	< 0.25 - 128	Zhao et al., 2018
Haemophilus parasuis	Pig	Diseased	110	93.6			0.008 - 16	Zhou et al., 2010
Helicobacter suis	Various (Pig, monkey)	N/A	35			5.7	0.03 - 32	Berlamont et al., 2019
Klebsiella pneumoniae	Cattle	Diseased		S			N/A	Arya et al., 2020
Pasteurella multocida	Rabbit	Diseased	10	100.0			0.0008 - 0.5	Sitovs et.al. 2023
Pseudomonas spp.	Buffalo	Diseased	3	66.6			N/A	Bhadaniya et al., 2019
Pseudomonas aeruginosa	Dog	Healthy	38			13.2	0.015 - 32	Park et al., 2020
Pseudomonas aeruginosa	Dog	Diseased	46			15.2	0.015 - 32	Park et al., 2020
Pseudomonas aeruginosa	Dog	Diseased	106			16.0	0.015 - 32	Rubin et al., 2008
Pseudomonas aeruginosa	Dog	Diseased	27	100.0	0.0	0.0	N/A	Ledbetter et al., 2007
Pseudomonas aeruginosa	Mink	Dead	69			13.0	16 - 128 (R)	Bai et al., 2019
Pseudomonas aeruginosa	Mink	Diseased/dead	30			13.3	N/A	Qi et al., 2014
Pseudomonas aeruginosa	Chicken	N/A	33	100.0	0.0	0.0	N/A	Eraky et al., 2020
Pseudomonas aeruginosa	Chicken	Diseased/dead	42	73.8	7.2	19.0	N/A	Farghaly et al., 2017
Proteus mirabilis	Various (Dog, cat)	Diseased	107		0.0	7.5	N/A	Marques et al., 2019
Proteus mirabilis (BP)	Various (Dog, mink, cattle, fowl)	Diseased	162	57.4	18.5	24.1	N/A	Sun et al., 2020
Proteus mirabilis (NBP)	Various (Dog, mink, cattle, fowl)	Diseased	14	57.1	0.0	42.9	N/A	Sun et al., 2020
Proteus mirabilis	Turtle	N/A	15	73.0	20.0	7.0	0.03 - 8	Pathirana et al., 2018
Proteus vulgaris	Turtle	N/A	7	85.7	14.3	0.0	0.03 - 4	Pathirana et al., 2018
Proteus hauseri	Turtle	N/A	2	100.0			0.06	Pathirana et al., 2018
Proteus vulgaris	Human (Catfish wound)	Diseased	1	100.0			< 0.25	Huang et al., 2013

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Shigella sonnei	Yak	Diseased	44			9.1	N/A	Zhu et al., 2018
Salmonella typhimurium	Guinea pig	N/A	35	60.0	14.3	24.7	N/A	Huamán et al., 2020
Salmonella spp.	Poultry	N/A	30		3.3	93.3	N/A	Tamuly et al., 2008
Salmonella spp.	Chicken (Broiler)	Diseased	5	60.0	40.0		N/A	Badr et al., 2020
Salmonella spp.	Chicken	N/A	19	78.9		15.8	N/A	Elfeil et al., 2020
Salmonella spp.	Duck	Diseased, dead	19	S			N/A	Rahman et al., 2016
Salmonella spp.	Pigeon	Diseased, dead	12	S			N/A	Rahman et al., 2016
Salmonella spp.	Pigeon	Healthy	11			18.2	N/A	Karim et al., 2020
Vibrio vulnificus	Seal	Diseased	1	S			N/A	Li et al., 2018
Vibrio spp.	Horse mackerel	N/A	9	100.0			N/A	Özer et al., 2008
Various (19 species; mostly *P. mirabilis*)	Owl monkey	Healthy	N/A	100.0			0.12	Da Silva et al., 2013

Table A3

Susceptibility of Gram-positive and atypical microorganisms isolated from animals to levofloxacin

MRSP – Multidrug-resistant *Staphylococcus pseudintermedius*, MRSA - methicillin-resistant *Staphylococcus aureus*, MDR – multidrug resistant, N – number of isolates, S – susceptible, I – intermediate, R – resistant, MIC – minimal inhibitory concentration, N/A – data not available in the reference source, iso – isolate

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Various	Cattle	Healthy, diseased	31	87.1			N/A	Bajaj et al., 2018
Trueperella pyogenes	Cattle	Diseased	100	100.0			<0.12 - 8	Fujimoto et al., 2023
Trueperella pyogenes	Pig	Diseased	67	100.0			<0.12 - 8	Fujimoto et al., 2023
Staphylococcus spp.	Cattle	Diseased	53			< 5.0	N/A	Zdolec et al., 2016
Staphylococcus spp.	Cattle	Healthy	41			< 5.0	N/A	Zdolec et al., 2016
Staphylococcus spp.	Cattle	Diseased	68	88.2	8.8	2.9	N/A	Mohanty et al., 2013
Staphylococcus spp.	Buffalo	Diseased	15	66.6			N/A	Bhadaniya et al., 2019
Staphylococcus pseudintermedius (MRSP)	Various (dog, cat, horse, donkey)	Diseased	146	2.1	0.0	97.9	<1 - 4	Ruscher et al., 2010
Staphylococcus pseudintermedius	Dog	Healthy, diseased	49			34.7	N/A	Kang et al., 2014
Staphylococcus pseudintermedius (MRSP)	Dog	Healthy, diseased	18			100.0	8 - >8	Sasaki et al., 2007
Staphylococcus intermedius	Dog	Healthy, diseased	114	98.2			N/A	Vanni et al., 2009
Staphylococcus schleiferi	Dog	Healthy, diseased	8	37.5			N/A	Vanni et al., 2009
Staphylococcus aureus	Dog	Diseased	6	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus	Pig	N/A	2		50.0	50.0	N/A	Sharma et al., 2020
Staphylococcus aureus	Cattle	Diseased	28	82.1	10.7	7.1	N/A	Sharma et al., 2020
Staphylococcus aureus	Buffalo	Diseased	21	81.0	19.0		N/A	Sharma et al., 2020
Staphylococcus aureus	Goat	Diseased	28	92.9	7.1		N/A	Sharma et al., 2020
Staphylococcus aureus	Sheep	Diseased	6	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus	Camel	Diseased	8	62.5	37.5		N/A	Sharma et al., 2020
Staphylococcus aureus	Horse	Diseased	3	100.0			N/A	Sharma et al., 2020
Staphylococcus aureus (MRSA ST 398)	Various (rabbit, human)	N/A	7	100.0			0.25 - 0.5	Agnoletti et al., 2014
Staphylococcus aureus (MRSA ST 398)	Pig	N/A	7	S (5 iso)	I (2 iso)		N/A	Lozano et al., 2011
Staphylococcus aureus (MRSA ST 793)	Pig	N/A	1			R (1 iso)	N/A	Lozano et al., 2011
Staphylococcus aureus (MDR)	Cattle	Diseased	48	S			N/A	Salauddin et al., 2020
Staphylococcus aureus	Cattle	Healthy	11	S			N/A	Tanzin et al., 2016
Staphylococcus aureus	Buffalo	Healthy	1	S			N/A	Tanzin et al., 2016
Staphylococcus aureus	Cattle	Diseased	20	S			N/A	Upadhyay and Kataria, 2009

Table A3 continued

Bacteria	Animal species	Health status	N	S %	I %	R %	MIC	Reference
Staphylococcus aureus	Goat	Diseased	10	S			N/A	Upadhyay and Kataria, 2009
Staphylococcus aureus	Goat	Healthy	32	84.4		15.6	N/A	Zhou et al., 2017
Staphylococcus aureus	Horse	Healthy	2	S			N/A	Van den Eede et al., 2013
Enterococcus faecium	Giant panda	Healthy	28			100.0	N/A	Liu et al., 2023
Enterococcus spp.	Cattle	N/A	176		0.0	0.0	N/A	Davedow et al., 2020
Lactobacillus spp.	Poultry (Indigenous)	N/A	59			81.4	32 - >128	Saleem et al., 2018
Lactobacillus spp.	Poultry (commercial)	N/A	46			97.8	32 - >128	Saleem et al., 2018
Actinomyces bowdenii	Dog	Diseased	1			R	N/A	Sherman et al., 2013
Streptococcus spp.	Cattle	Diseased	46	89.1	6.5	2.2	N/A	Mohanty et al., 2013
Streptococcus spp.	Buffalo	Diseased	1	100.0			N/A	Bhadaniya et al., 2019
Streptococcus agalacticae	Elephants (captive)	Diseased	25	100.0			<1	Eisenberg et al., 2017
Streptococcus agalacticae	Cattle	Diseased	133	18.1	18.1	63.9	N/A	Yang et al., 2020
Streptococcus suis	Pig	Diseased	16	100.0			0.25 - 1	Ichikawa et al., 2020
Streptococcus suis	Pig	Healthy, diseased	98	100.0			0.5 - 4	Ichikawa et al., 2020
Streptococcus suis	Pig	Healthy	260	62.3	6.2	31.5	N/A	Soares et al., 2014
Clostridium difficile	Dog (puppy)	Healthy	34			100.0	>32	Álvarez-Pérez et al., 2014a
Clostridium difficile	Cattle (beef)	N/A	94			100.0	2 - >32	Thitaram et al., 2016
Clostridium difficile	Cattle (dairy)	N/A	188			96.8	2 - >32	Thitaram et al., 2016
Clostridium difficile	Pig	N/A	94			100.0	2 - >32	Thitaram et al., 2016
Clostridium difficile	Cattle (calf)	Healthy, diseased	30			73.0	4 - >32	Rodriguez-Palacios et al., 2006
Clostridium difficile	Cattle	N/A	103				<2 - 16	Bandelj et al., 2017
Clostridium difficile	Pig	Healthy	41			100.0	>32	Álvarez-Pérez et al., 2013
Clostridium difficile	Zebra	Healthy	4			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Goat	Healthy	1			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Iberian ibex	Healthy	1			100.0	>32	Álvarez-Pérez et al., 2014b
Clostridium difficile	Chimpanzee	Diseased	1			100.0	>32	Álvarez-Pérez et al., 2014b
Bacillus spp.	Buffalo	Diseased	3	66.6			N/A	Bhadaniya et al., 2019
Micrococcus spp.	Buffalo	Diseased	9	88.8			N/A	Bhadaniya et al., 2019
Corynebacterium spp.	Buffalo	Diseased	11	90.9			N/A	Bhadaniya et al., 2019
Mycoplasma bovis	Cattle (beef, dairy)	N/A	26	S			0.5 - 2	Mustafa et al., 2013
Mycobacterium avium subsp.hominissuis	Cat	Diseased	1			R	1	Kanegi et al., 2019

Table A4

Main levofloxacin pharmacokinetic parameters (±SD) reported in mammals after a single administration (unless otherwise noted)

SD – standard deviation, ROA – route of administration, IV – intravenous, IM – intramuscular, SC – subcutaneous, IP – intraperitoneal, PO – oral, BW – body weight, SR – sustained release, inf – infusion, Cl – plasma clearance, $T_{1/2el}$ – half-life of elimination, Vdss – volume of distribution at steady state, F – bioavailability, N/A – data not available in the reference source *Median value (range)

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	$T_{1/2el}$ (h)	Vdss (mL/g)	F%	Reference
Dog	IV	5	0.29 ± 0.09	7.93 ± 1.41			Urzúa et al., 2020
	PO	5		7.65 ± 1.38		72 ± 10	
	IV	15	0.15 ± 0.03	6.23 ± 0.91	1.19 ± 0.20		Madsen et al., 2019
	PO	23.7		5.84 ± 1.17		104 ± 30	
	IV	2.5	0.11 ± 0.03	7.85 ± 2.30	1.20 ± 0.13		Landoni et al., 2018
	SC	5		7.78 ± 1.55		80 ± 8	
	PO	5.6		6.01 ± 1.32		61 ± 15	
	PO 300mg			4.92 ± 1.94			Yin et al., 2011
	PO 300mg (SR 1)			7.15 ± 2.13		42 ± 5	
	PO 300mg (SR 2)			8.40 ± 1.01		103 ± 4	
Cat	IV	10	0.14 ± 0.04	9.31 ± 1.63	1.75 ± 0.42		Albarellos et al., 2005
	PO (4 days mean)	10		8.39 ± 2.14		86 ± 44	
Asian elephant	PO	5		12.11 ± 1.45			Kilburn et al., 2022
	Rectal	15		10.16 ± 1.41		64 ± 129	
Giant panda	IM	2		5.40 (0.70)*			Wang et al., 2021
	PO	3		7.14 (0.63)*			
Rabbit	IV	5	0.60 ± 0.18	2.06 ± 0.18	1.37 ± 0.39		Sitovs et al., 2020
	IM	5		2.01 ± 0.24		106 ± 28	
	SC	5		1.80 ± 0.14		119 ± 41	
	IV (30 min inf)	20	1.7 (L/h)	3.99 ± 0.92			Czyrski et al., 2014
Rabbit (Meningitis model)	IV (10 min inf)	7		7.60 ± 3.50			Destache et al., 2001
	IV (10 min inf)	10.5		7.00 ± 1.60			
	IV (10 min inf)	14		9.50 ± 3.50			
Guinea pig (Pneumonia model)	IP	10		1.00 ± N/A			Edelstein et al., 1996
Rat	IV	7	0.21 (L/h)	5.00 ± 1.70	1.20 ± 0.40		Hurtado et al., 2014
	PO	100		1.76 ± N/A			Dharuman et al., 2010
	IV	3					Cheng et al., 2002
Mouse	PO	10		5.65 ± 0.14			Yarsan et al., 2003
Mouse (Toxoplasmosis model)	PO	10		4.54 ± 0.50			Yarsan et al., 2003

Table A4 continued

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	T1/2el (h)	Vdss (mL/g)	F%	Reference
Cattle (calf)	IV	10	0.34 ± 0.01	2.12 ± 0.21	0.98 ± 0.10		Kumar et al., 2012
	IM	10		2.76 ± 0.36		63 ± 6	
Cattle (crossbred calf)	PO	20		2.99 ± 0.15			Kumar et al., 2009
Cattle (crossbred calf; febrile)	PO	20		3.05 ± 0.16			
Cattle (crossbred calf)	IV	4	0.32 ± 0.05	1.61 ± 0.07	0.74 ± 0.03 (V_{area})		Dumka and Srivastava, 2007
	IM	4		3.67 ± 0.40		57 ± 12	Dumka and Srivastava, 2006
Buffalo (calf)	IM	3		3.27 ± 0.31		68 ± 5	Ram et al., 2008
Goat (non-lactating)	IV	2	0.46 ± 0.11	4.56 ± 1.24	1.22 ± 0.22		Vercelli et al., 2020
	SC	2		5.14 ± 0.57		92 ± 59	
Goat	IV	10	0.34 ± 0.05	4.04 ± 0.24	1.89 ± 0.18 (V_{area})		Ram et al., 2011
Goat (Mastitis model)	IV	10	0.35 ± 0.03	5.08 ± 0.18	2.56 ± 0.21 (V_{area})		
Goat (lactating)	IV	4	0.18 ± 0.04	2.95 ± 0.27	0.73 ± 0.22		Goudah and Abo-El-Sooud, 2009
	IM	4		3.64 ± 0.42		85 ± 8	
Sheep	IV	2	0.19 ± 0.02	4.06 ± 2.41	0.56 ± 0.18		Sartini et al., 2020a
	PO (5 days)	2		3.76 ± 1.73		115 ± 28	
	IV	4	0.39 ± 0.04	1.82 ± 0.05	0.96 ± 0.08		Durna Corum et al., 2020
	IV	3	0.55 ± 0.02	2.38 ± 0.22	0.92 ± 0.08		Patel et al., 2012
	SC	3		1.73 ± 0.04		91 ± 4	
	IV	4	0.20 ± 0.05	3.29 ± 0.23	0.86 ± 0.23		Goudah and Hasabelnaby, 2010
	IM	4		3.58 ± 0.30		91 ± 7	
Camel	IV	4	0.28 ± 0.03	2.92 ± 0.61	1.01 ± 0.36		Goudah, 2008
	IM	4		3.47 ± 0.86		94 ± 8	
Horse (Stallion)	IV	4	0.21 ± 0.18	2.58 ± 0.51	0.81 ± 0.26		Goudah et al., 2008
	IM	4		2.94 ± 0.78		92 ± 13	
Marmoset	PO	40		3.90 ± N/A			Nelson et al., 2010
	PO (7 days)	40		2.30 ± N/A			
Rhesus monkey (Anthrax model)	PO	15		2.10 ± 0.12			Kao et al., 2006
	PO	25		1.86 ± 0.28			
Rhesus monkey (male)	PO (C14-labelled)	15		1.67 ± N/A			Hemeryck et al., 2006
Rhesus monkey (female)	PO (C14-labelled)	15		1.90 ± N/A			

Table A5

Main levofloxacin pharmacokinetic parameters (±SD) reported in birds after a single administration (unless otherwise noted)

Species	ROA	Dose (mg/kg BW)	Cl (mL/g/h)	$T_{1/2el}$ (h)	Vdss (mL/g)	F%	Reference
Poultry (not specified)	IM (5 days)	10		2.97 ± 0.11			Bisht et al., 2018
Chicken (broiler)	IV	5	0.38 ± 0.09	6.93 ± 2.94	2.88 ± 1.07		Lee et al., 2017
	PO	5		8.09 ± 1.71		123 ± N/A	
	IV	10	0.44 ± 0.01	4.07 ± 0.24	2.36 ± 0.13		El-Banna et al., 2013
	PO	10		4.24 ± 0.28		107 ± 9	
Chicken (Leghorn bird)	IV	10	0.25 ± 0.00	3.08 ± 0.05	3.23 ± 0.06		Patel et al., 2012b
	PO	10		3.62 ± 0.12		72 ± 1	
Chicken (broiler)	IV	10	0.25 ± 0.00	3.18 ± 0.07	3.25 ± 0.06		Varia et al., 2009
	PO	10		3.64 ± 0.15		60 ± 2	
Turkey	IV	10	0.23 ± 0.03	4.49 ± 0.12	1.31 ± 0.04		Aboubakr et al., 2012
	IM	10		4.60 ± 0.22		96 ± 4	
	PO	10		4.07 ± 0.17		80 ± 3	
Quail (Japanese)	IV	10	0.40 ± 0.03	2.52 ± 0.07	1.27 ± 0.06		Aboubakr, 2012
	PO	10		2.83 ± 0.30		69 ± 2	
Geese (Bilgorajska)	IV	2	0.28 ± 0.06	7.39 ± 1.21	1.40 ± 0.28		Sartini et al., 2020b
	PO	5		6.60 ± 2.46		96 ± 21	
Duck (Muscovy)	IV	10	0.41 ± 0.04	2.76 ± 0.10	1.37 ± 0.07		Aboubakr and Soliman, 2012
Duck (Muscovy; renal damage)	IV	10	0.20 ± 0.02	4.71 ± 0.54	1.18 ± 0.04		
Duck (Muscovy)	PO	10		2.89 ± 0.09		74 ± 2	
Duck (Muscovy; renal damage)	PO	10		3.94 ± 0.14		72 ± 2	

SD – standard deviation, ROA – route of administration, IV – intravenous, IM – intramuscular, PO – oral, BW – body weight, Cl – plasma clearance, $T_{1/2el}$ – half-life of elimination, V_{dss} – volume of distribution in steady state, F – bioavailability, N/A –data not available in the reference source

Table A6

Average levofloxacin plasma protein binding (± SD)

Mammals	**Protein binding %**	**Reference**
Dog	23.7 ± 3.8	Madsen et al., 2019
Rabbit	25.0 ± N/A	Destache at al., 2001
Rat	45.5 ± 9.4	Hurtado et al., 2014
Cattle (crossbred calf)	17.0 ± 1.2	Dumka and Srivastava, 2006
Buffalo (calf)	19.1 ± 1.5	Ram et al., 2008
Goat	Range: 23.0 – 34.8	Ram et al., 2011
Goat (lactating)	22.0 ± N/A	Goudah and Abo-El-Sooud, 2009
Sheep	23.7 ± N/A	Goudah and Hasabelnaby, 2010
Camel	23.5 (Range 21.0 – 27.0)	Goudah, 2008
Horse (stallion)	27.8 (Range 20.0 – 29.0)	Goudah et al., 2008
Rhesus monkey	11.2 ± N/A	Hemeryck et al., 2006
Birds	**Protein binding %**	**Reference**
Chicken (broiler)	24.0 ± 5.0	Lee et al., 2017
Chicken (broiler)	4.2 ± 0.5	El-Banna et al., 2013
Turkey	24.3 ± N/A	Aboubakr et al., 2014
Quail (Japanese)	23.0 ± N/A	Aboubakr, 2012

SD – standard deviation, N/A –data not available in the reference source

Table A7

Tissue disposition and suggested withdrawal times of levofloxacin in poultry

Species	ROA	Dose (mg/kg)	C_{max} (µg/kg)	t_{max}	t_{last}	PCO	Tissues analysed	S WT	Reference
Chicken	PO	10	1051	0d	10d	Liver	Muscle, liver, gizzard, heart, skin	N/A	Kyuchukova et al., 2013
Chicken (broiler)	PO	10	9330	2h	9d	Kidney	Muscle, liver, kidney, lung, fat, spleen	> 9 days	El-Banna et al., 2013
Chicken	PO	10	1429	1d	10d	Liver	Muscle, liver	4 days	Ravikumar et al., 2016
Chicken (broiler)	PO	5	657	1h	48h	Liver	Muscle, liver, kidney, lung	N/A	Lee et al., 2017
Chicken	PO	10	1222	1 d	10d	Liver	Muscle, liver	5 days	Suman 2018
Chicken	PO	20	2251	1 d	10d	Liver	Muscle, liver	5 days	Suman 2018
Poultry	IM	10	140	24h	72h	Kidney	Muscle, liver, kidney	N/A	Bisht et al., 2018
Geese (Bilgorajska)	PO	5	642	6h	48h	Liver	Muscle, liver, lung, kidney, heart	90 h	Sartini et al., 2020b

ROA – route of administration, IV – intravenous, IM – intramuscular, PO – oral, C_{max} – maximum detected levofloxacin concentration, t_{max} – time of maximum detected levofloxacin concentration, T_{last} – last detectable levofloxacin concentration, S WT – suggested withdrawal time, PCO – organ or tissue where maximum levofloxacin concentration was detected, N/A – data not available in the reference source