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ORIGINAL ARTICLE

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 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

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

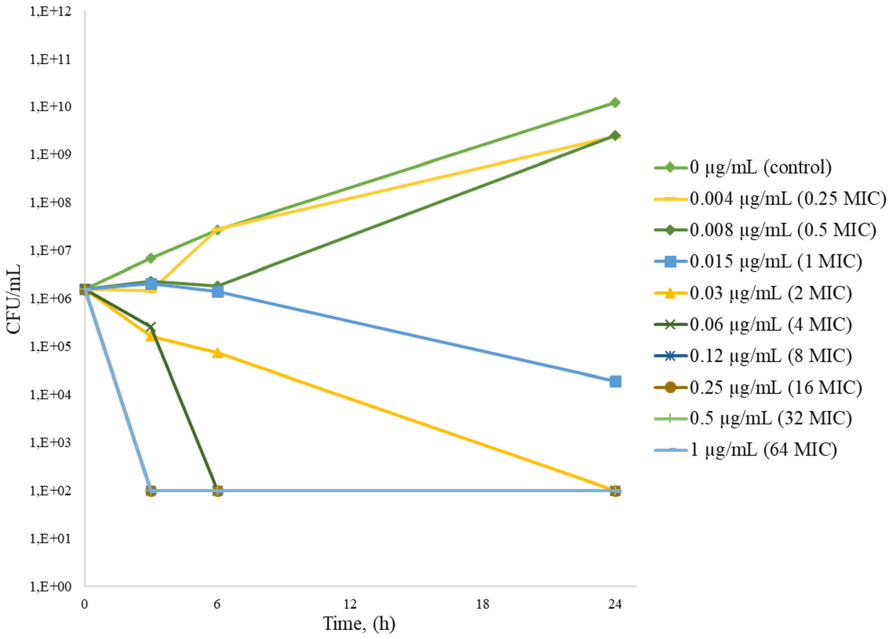
[a]*P. multocida* isolate selected for in vitro and ex vivo bacterial time-killing study.

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*

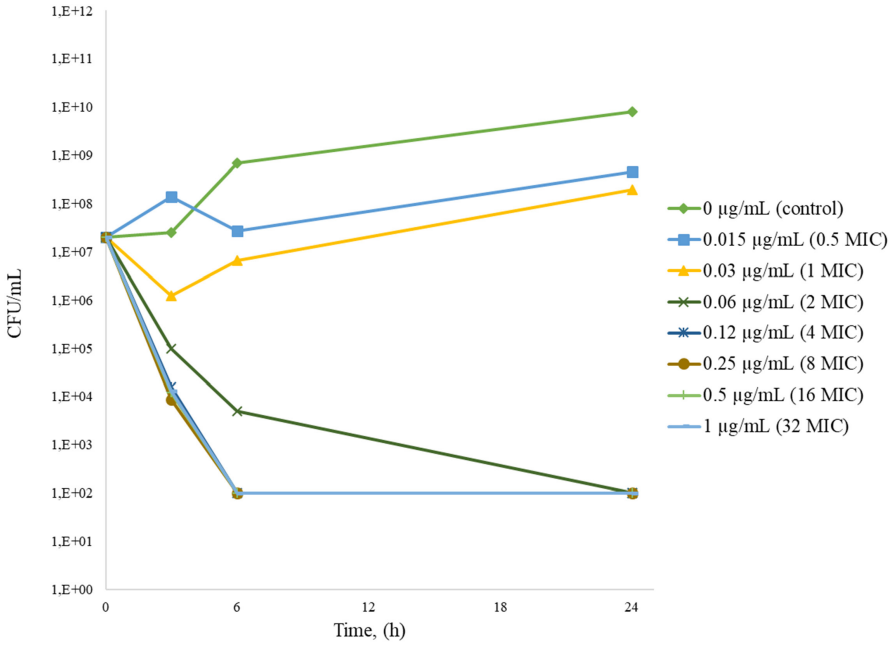


FIGURE 2 In vitro time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in rabbit serum. Standard error bars are excluded for clarity.

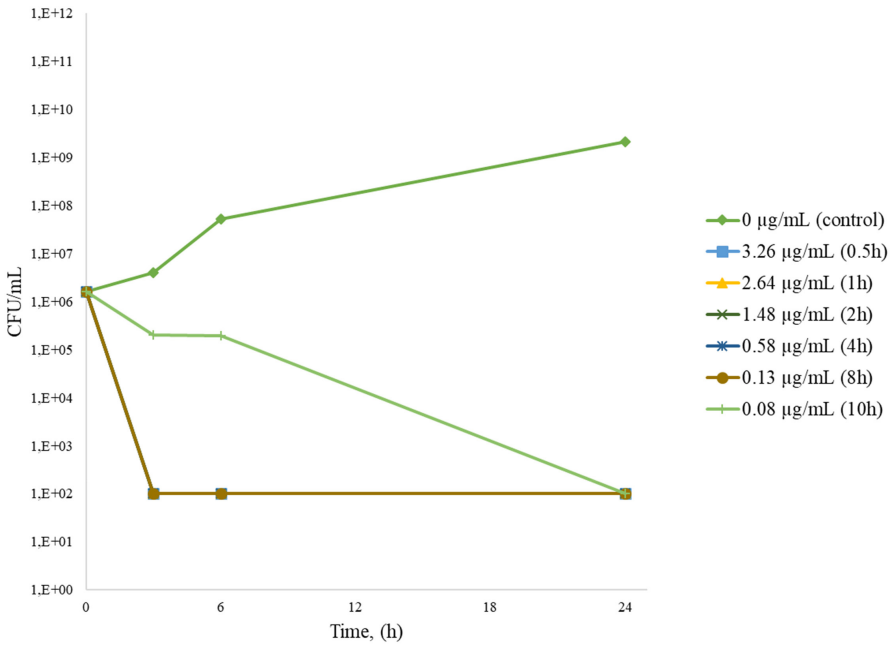


FIGURE 3 Ex vivo time-killing curves representing the growth of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 µg/mL) with different levofloxacin concentrations in serum samples obtained after intramuscular dose of 5 mg/kg to healthy rabbits ($n = 6$). Standard error bars are excluded for clarity.

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

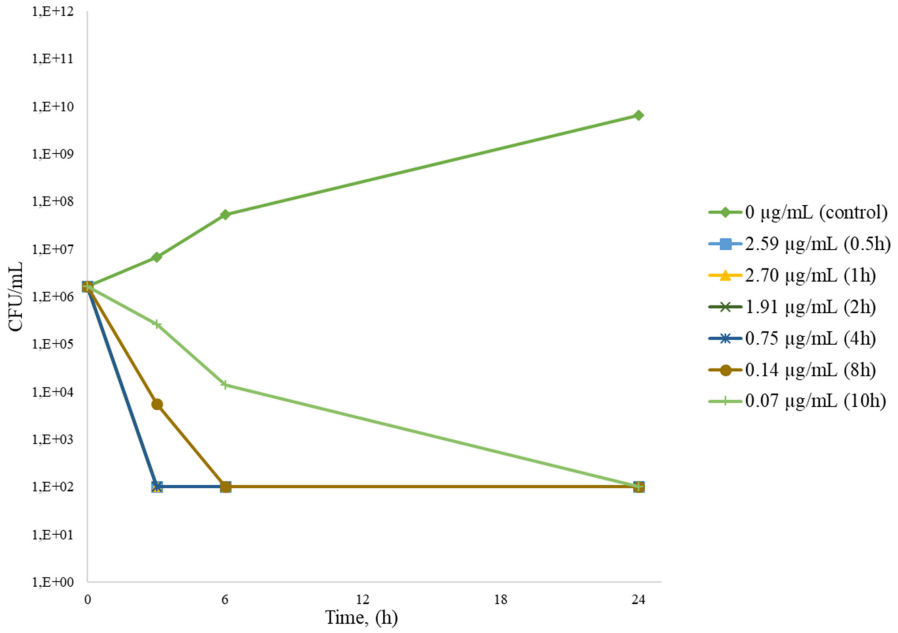


FIGURE 4 Ex vivo time-killing curves representing the growth of *Pasteurella multocida* (Nr. 7697, MIC = 0.015 µg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous dose of 5 mg/kg to healthy rabbits ($n = 4$). Standard error bars are excluded for clarity.

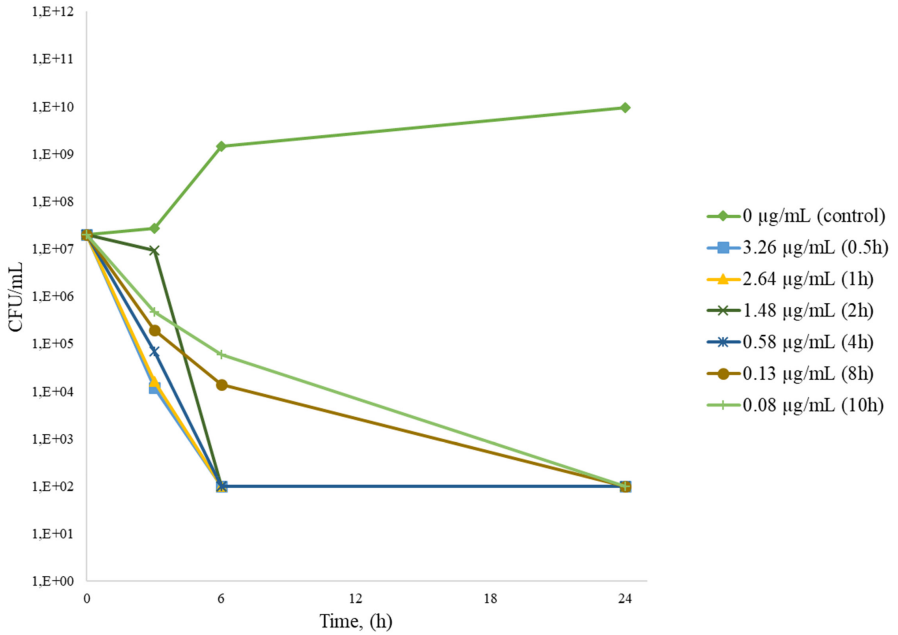


FIGURE 5 Ex vivo time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 µg/mL) with different levofloxacin concentrations in samples obtained after intramuscular dose of 5 mg/kg to healthy rabbits ($n = 6$). Standard error bars are excluded for clarity.

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

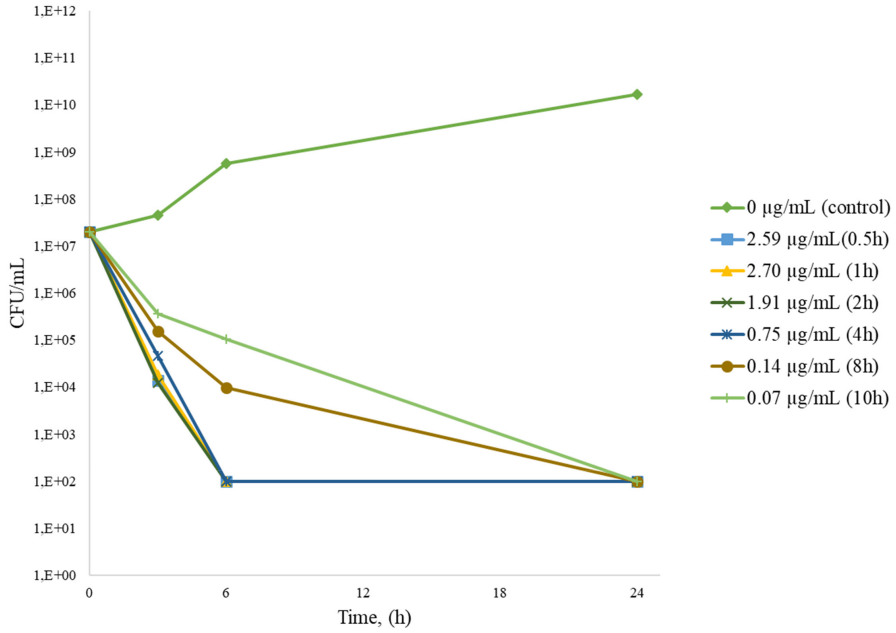


FIGURE 6 Ex vivo time-killing curves representing the growth *Escherichia coli* (Nr. 1, MIC = 0.03 μg/mL) with different levofloxacin concentrations in samples obtained after subcutaneous dose of 5 mg/kg to healthy rabbits ($n = 4$). Standard error bars are excluded for clarity.

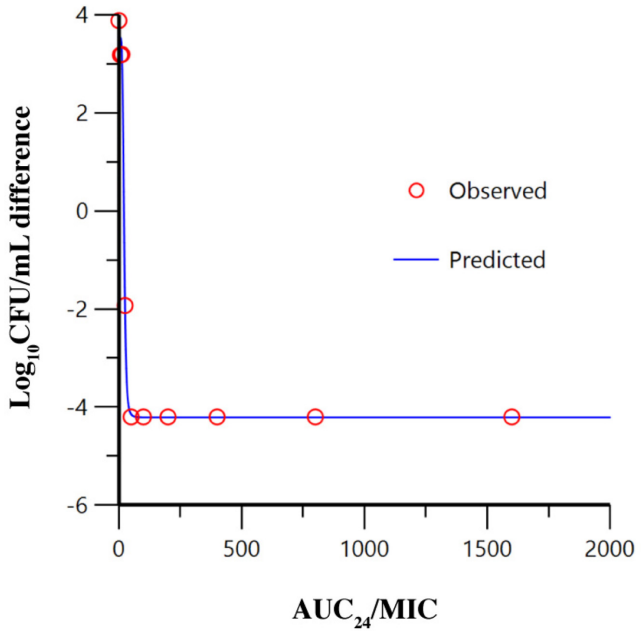


FIGURE 7 Plot of in vitro AUC_{24}/MIC versus *Pasteurella multocida* (Nr. 7697, MIC = 0.015 μg/mL) bacterial count difference in levofloxacin containing rabbit serum.

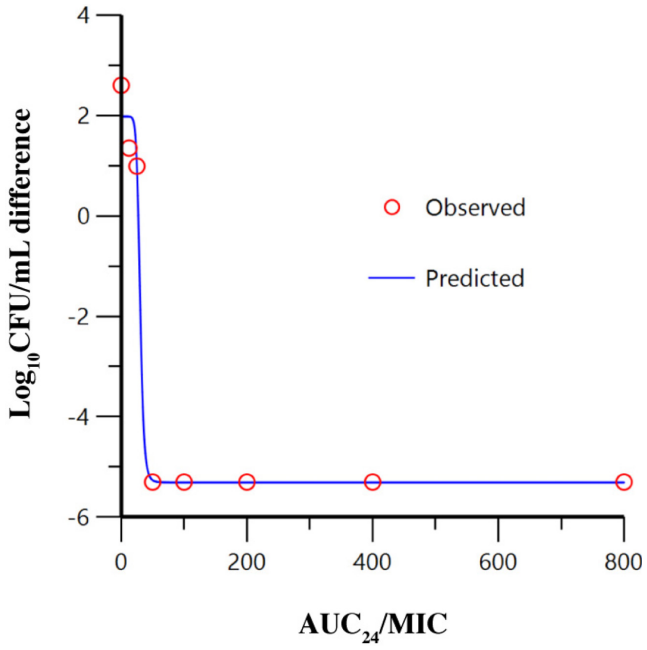


FIGURE 8 Plot of in vitro AUC_{24}/MIC versus *Escherichia coli* (Nr. 1, MIC = 0.03 μg/mL) bacterial count difference in levofloxacin containing rabbit serum.

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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