Pharmacokinetics of gallium maltolate in *Lawsonia intracellularis*-infected and uninfected rabbits

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Oral gallium maltolate (GaM) pharmacokinetics (PK) and intestinal tissue (IT) concentrations of elemental gallium ([Ga]) and iron ([Fe]) were investigated in a rabbit model of equine proliferative enteropathy (EPE). New Zealand white does (uninfected controls and EPE-infected, $n = 6$/group) were given a single oral GaM dose (50 mg/kg). Serial blood samples were collected from 0 to 216 h post-treatment (PT) and IT samples after euthanasia. Serology, qPCR, and immunohistochemistry confirmed, or excluded, EPE. Blood and IT [Ga] and [Fe] were determined using inductively coupled plasma–mass spectrometry. PK parameters were estimated through noncompartmental approaches. For all statistical comparisons on [Ga] and [Fe] $\alpha = 5\%$. The Ga log-linear terminal phase rate constant was lower in EPE rabbits vs. uninfected controls [0.0116 ± 0.004 (SD) vs. 0.0171 ± 0.0028 per hour; $P = 0.03$]; but half-life (59.4 ± 24.0 vs. 39.4 ± 10.8 h; $P = 0.12$); C_{max} (0.50 ± 0.21 vs. 0.59 ± 0.42 µg/mL; $P = 0.45$); t_{max} (1.75 ± 0.41 vs. 0.9 ± 0.37 h; $P = 0.20$); and oral clearance (6.743 ± 1.887 vs. 7.208 ± 2.565 L/h; $P = 0.74$) were not. IT's [Ga] and [Fe] were higher ($P < 0.0001$) in controls. In conclusion, although infection reduces IT [Ga] and [Fe], a 48 h GaM dosing interval is appropriate for multidose studies in EPE rabbits.

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INTRODUCTION

Equine proliferative enteropathy (EPE) is an emerging disease in weanling horses caused by *Lawsonia intracellularis,* an obligate intracellular gram-negative bacterium (McOrist *et al.*, 1995a; Pusterla & Gebhart, 2009). The disease causes hyperplasia of enterocytes and enteric crypts, primarily in the distal jejunum and ileum, and occasionally in the cecum and colon, affecting the intestinal ability to absorb nutrients in multiple domestic and wildlife animal species (Herbst *et al.*, 2003; Horiuchi *et al.*, 2008; Pusterla & Gebhart, 2009; Wong *et al.*, 2009). In weanling foals, EPE's clinical signs range in severity and chronicity from generalized wasting, pronounced ventral edema (along with hypoproteinemia), to profuse diarrhea, fever, colic, acute shock, and death (Lavoie *et al.*, 2000; Pusterla & Gebhart, 2009; Page *et al.*, 2012). Similarly to its impact on the swine production industry worldwide, *L. intracellularis* infection in weanling horses is a significant economic and welfare burden for the equestrian industry, with economic losses due to animal mortality, poor growth rates, and costly therapeutic interventions. Moreover, the long convalescence and recovery associated with EPE constitute a sale-price drawback, up to a year after clinical manifestation (Guedes *et al.*, 2002a; Frazer, 2008).

Lawsonia intracellularis infections pose a challenge for antimicrobial drug (AMs) therapy due to the bacteria's privileged intracellular location within enterocytes. This 'sanctuary-like' location protects the bacteria from the host defenses and from the common first-line AMs adopted by equine clinicians. Currently, the therapeutic management of EPE relies on the oral employment of macrolides or azalides, often combined with rifampin, or the tetracycline class, either through parenteral or oral dose (Lavoie *et al.*, 2000; Schumacher *et al.*, 2000; Sampieri *et al.*, 2006; Feary *et al.*, 2007; Frazer, 2008). No new alternative AMs have been proposed for EPE therapy since 2006. A few reported cases of treatment failures suggest a

need for alternative therapeutic approaches, but the potential for *L. intracellularis* to develop a specific susceptibility pattern, or even an AM resistance, although currently unknown, provides increasing motivation to identify other AM treatments (Frazer, 2008). Strain susceptibility studies *in vitro* have not yet been conducted on the EPE strain. For the porcine proliferative enteropathy (PPE) strains, the limited number of studies conducted thus far suggests a potential for geographical strain differentiation (McOrist *et al.*, 1995b; Wattanaphansak *et al.*, 2009a). Interestingly, most therapeutic knowledge has been achieved through clinical experiences, rather than laboratory trials, due to intrinsic difficulties in maintaining *L. intracellularis* in a pure cell-culture environment (Lawson *et al.*, 1993). A few studies involving exposure of cultured bacteria *in vitro* to a variety of AMs showed the clear technical limitations of *in vitro* research for intracellular organisms (Wattanaphansak *et al.*, 2009a,b). To overcome these limitations, in this study, the use of a rabbit infection model for EPE, started from an equine strain of *L. intracellularis,* was elected (Sampieri *et al.*, 2013), to obtain a valuable preclinical characterization of the drug during disease, for the needs of EPE therapy.

A potential alternative AM therapy for EPE is the novel, gallium-based compound, gallium maltolate (GaM). It is hypothesized that gallium may reach intracellular bacteria through typical mechanisms of oligo-element absorption (specifically iron, zinc and aluminum) in the small intestine (Gunther & Wright, 1983; Caspary, 1992). Given its similarity in electric charge, ionic radius, valence and electronic footprint to ferric iron (FeIII), elemental gallium (GaIII) may function through a 'Trojan horse' mechanism replacing FeIII, a metabolically essential element to most pathogens' survival, and thereby reduce the risk for development of AM resistance (Bernstein, 1998; Collery *et al.*, 2002). Gallium is predominantly detected in plasma bound to FeIII-sites of transferrin. It is preferentially taken up by phagocytic cells during the inflammation process, entering such cells through mechanisms dependent and independent of transferrin (Tsan, 1986; Chitambar & Zivkovic, 1987). Selectivity must occur because bacteria take up ferric iron, while healthy mammalian cells tend to heavily recycle iron locally or, in the case of erythropoietic cells, take up ferrous iron (FeII), which subsequently undergoes intracellular redox reactions (Logan *et al.*, 1981; Bernstein, 1998). In the presence of sufficiently high GaIII concentration, then, bacteria may take up large amounts of GaIII, altering the first step of the bacterial ferric metabolism (Olakanmi *et al.*, 2000). Unlike iron, gallium is unable to complete a redox-cycling reaction (from valence III to valence II) and thus cannot be incorporated, or function, in many iron metabolic pathways and enzymes (e.g., ribonucleotide reductase) necessary for DNA replication and cell function (Bernstein, 1998). The ability of GaIII to block these iron-dependent metabolic steps has been demonstrated in *Mycobacteria* and hypothesized in *R. equi,* and it has been clearly ascribed to the gallium moiety, not to the particular salt or ligand utilized in the formulation (i.e., nitrate, chloride, or maltolate) (Olakanmi *et al.*, 2000; Harrington *et al.*, 2006). Inhibition of bacterial iron

metabolism may lead to AM effects, as it may ultimately reduce bacterial survival and replication (Bernstein, 1998).

The AM activity of several gallium-based compounds has been explored for a few intracellular bacteria in veterinary medicine (Harrington *et al.*, 2006; Martens *et al.*, 2007; Fecteau *et al.*, 2011). Only an injectable solution of citrate-chelated gallium nitrate is approved in United States for the treatment for human cancer-related hypercalcemia and is administered as a continuous i.v. infusion, to avoid the nephrotoxic effects reported with i.v. bolus (Martens *et al.*, 2007). GaM is deemed safe when orally administered to humans, mice, rats, dogs, and foals (Bernstein *et al.*, 2000; Martens *et al.*, 2010). Unlike with gallium salts (i.e., gallium chloride or nitrate), there are no reports of GaM nephrotoxicity, as it is apparently highly protein-bound in the bloodstream; it is thus expected to convey GaIII to the bacteria located in the intestinal brush border membrane (Collery *et al.*, 1989; Bernstein *et al.*, 2000).

In this study, the potential differences induced by EPE on GaM pharmacokinetics (PK) parameters were investigated in rabbits, prior to exposing to treatment foals affected by *L. intracellularis*. This study aimed to collect sufficient information on the PK characteristics of this novel compound, to determine the feasibility of a multidosing GaM efficacy in a rabbit infection model for EPE (equine strain of *L. intracellularis*), using both the same bolus dose (50 mg/kg BW once intragastrically) previously utilized in a mouse model for *R. equi,* and the route of administration utilized in a preclinical trial in *R. equi* affected foals (Harrington *et al.*, 2006; Martens *et al.*, 2010; Chaffin *et al.*, 2011).

MATERIAL AND METHODS

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and it adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Animals

Eighteen, 8- to 9-week-old, 2.01–3.07 kg BW, New Zealand white female rabbits (Charles River Canada, Pointe Claire, QC, Canada) were used. Upon arrival, all does underwent a health check and identification, with a permanent marker felt-pen on the right ear. They were housed in the Animal Care Unit facilities at the University of Saskatchewan. The uninfected control group was housed in a Containment Level (CL)-1 room and the EPE-infected group in a CL-2 Room. Experiments started after a mandatory week of acclimation. All rabbits were fed *ad libitum* pellets (Co-op Whole Earth* Rabbit Ration; Federated Co-op. Ltd. Saskatoon, SK, Canada) and water, and maintained in standard husbandry conditions (12/12 h light/dark cycle, and 20 ± 2 °C room temperature). For all animals, environmental enrichment (toys and treats) was provided. Treats (apples and carrots) were offered to promote socialization, facilitate handling, and monitor individual appetite, alertness, and brightness, particularly in group-housing conditions (Johnson-Delaney, 2006; Sampieri

et al., 2013). Animals were monitored daily for body weight gain, and twice or more often (in the case of multiple samples in a day) for appetite, water intake, attitude, fecal consistency, and grooming, as previously reported (Sampieri *et al.*, 2013).

L. intracellularis rabbit infection model

The protocol described for inducing equine strain *L. intracellularis* lesions in juvenile rabbits was followed to achieve infection in the EPE-infected group (Sampieri *et al.*, 2013). Each rabbit was inoculated via nasogastric intubation with a low passage pure cell-culture inoculum of E04504 *L. intracellularis*, harvested after isolating the bacteria from a deceased foal's ileal mucosa. Inocula were prepared and suspended in 3 mL of buffered sucrose/phosphate/glutamate medium, for a total inoculating dose of 2.5×10^8 bacteria/rabbit (Sampieri *et al.*, 2013), administered with a chasing volume of 3 mL of distilled water. A SYBR Green-based PCR quantitation assay was used to determine the bacterial concentration of the infectious material, as previously published (Wattanaphansak *et al.*, 2010). Onset of infection, or its absence in the controls, was detected *ante mortem* through diagnostics used currently in clinical practice for EPE, such as immuneperoxidase monolayer assay (IPMA) serology and TaqMan qPCR on fecal material (Guedes *et al.*, 2002b; Sampieri *et al.*, 2013). The *post mortem* assessment was based on gross pathology examination, hematoxilin-eosin (H&E) staining, and immunohistochemistry (IHC) labeling of specific lesions, based on murine anti-*L. intracellularis* antibody, on samples fixed in 10% buffered formalin at collection time (Guedes *et al.*, 2002c).

Study design

Rabbits were divided into two groups of nine animals each: the uninfected control group (controls) and EPE-infected group (EPE-infected). In each group, three does were randomly selected for a pilot – limit of detection – experiment. Those rabbits underwent euthanasia and full necropsy 24 h post-treatment (PT).

The remaining six rabbits of both groups were enrolled in the PK study: the uninfected control and the EPE-infected groups were studied in two phases, a few days apart, so that only three animals at a time were treated, sampled and euthanized, to maximize accuracy in sample collection and animal monitoring, throughout the experiment.

Gallium maltolate (provided by LRB, Terrametrix, Menlo Park, CA, USA) was dissolved in sterile double-distilled water, immediately prior to dosing the animals, achieving a concentration of 8 mg/mL, with a solution's final pH = 5.8–6 (Harrington *et al.*, 2006). Rabbits were administered a single intragastric dose of GaM solution, 50 mg/kg BW, on day 0 for the controls and on day 7 postinfection (PI) for the EPE-infected rabbits. Such timing was elected to evaluate the ability of small intestine to absorb the solution when the EPE lesions start to appear, as the infection peak in the rabbit model is reported to be around 12–14 days PI (Sampieri *et al.*, 2013).

A nasogastric tube was used for intragastric dose of GaM, due to the relatively large administered volume. Briefly, an anesthetic gel (Xylocaine Gel 2%; Astra Zeneca Canada Inc., Missisauga, ON, Canada) was applied medially on both nostrils. Approximately 30–45 min later, a 5 Fr. wide, 40-cm-long feeding tube (Kendall Sovereign; Tyco Health Care Group LP, Mansfield, MA, USA) was inserted via the nose into the stomach. Serial blood samples were taken from a catheter inserted in the ear's central artery. Briefly, a local skin block above the ear's central artery was achieved with a minute amount of local anesthetic cream (EMLA Cream; Astra Zeneca Canada Inc., Missisauga, ON, Canada). The rabbits were placed within restraint cages and the catheterization of the ear's central artery was performed following a previously published protocol (Sampieri *et al.*, 2012), with an indwelling sterile catheter (BD Insyte – Becton Dickinson Infusion Therapy Systems Inc. Sandy, UT, USA) affixed to an injection port (Intermittent Infusion Plug – Kendall Argyle TYCO Healthcare Group LP. USA). The injection port, along with the catheter hub, was glued temporarily to the skin with tissue glue (3M Vetbond – 3M Animal Care Products, St. Paul, MN, USA). Subsequently, the whole ear was bandaged with moleskin (Dr. Scholl's Extra Soft Moleskin, imported Schering Canada Inc., Pointe Claire, QC, Canada) in such a way that the bandage mimicked a skin embedded port, affording timely repeated samples (Sampieri *et al.*, 2012).

Sample collection

Blood. Arterial blood was collected (1 mL/sample) from time 0 h (t_{first}), immediately after GaM dose, and then at time 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 h, and subsequently every 24 h, until time 216 h (9 days PT, t_{last}). The first nine samples were collected through the injection port bandaged in place, as described above. Starting with the sample at 24 h PT to the t_{last} sample, blood was collected off the needle, after local anesthesia was applied on the skin over the ear's central artery.

Tissue samples. A full necropsy examination was conducted in all 18 rabbits. A published, sampling protocol (Sampieri *et al.*, 2013) was followed for visual examination of the organs, from duodenum to rectum, for thickness, discoloration or type of content, as *L. intracellularis* lesions are not located in any other organ. Samples of duodenum, mid-jejunum and terminal jejunum, ileum, ileocecal valve (including *ampulla coli* and *sacculus rotundus*), cecum (transitional area between cecum and cecal appendix), terminal portion of the cecal *appendix*, large colon (proximal to the *fusum coli*), terminal colon, and rectum were collected (Sampieri *et al.*, 2013).

Verification of infection

Histology and immunohistochemistry. Two adjacent, formalin-fixed and paraffin-embedded sections per sample were cut and stained by H&E and IHC, with streptavidin method using murine anti-*L. intracellularis*-specific monoclonal antibody, to detect proliferative lesions of the intestinal epithelium and the

presence of the antigen within the cells (Guedes & Gebhart, 2003a). The *L. intracellularis*-specific antigen in the enterocytes was blindly evaluated with a five-grade IHC scoring system, as previously reported (Guedes & Gebhart, 2003b). For each rabbit, the negative control for each tissue section consisted of a correspondent IHC-labeled tissue section, except for the primary antibody. Furthermore, pig ileal tissues known to be negative and positive for *L. intracellularis* infection were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

Serology. In serum, anti-*L. intracellularis*-specific IgG concentration was measured by an IPMA as reported previously (Guedes *et al.*, 2002b). Positive serum samples were end-point titrated starting with a dilution of 1:30 up to 1:1920. Control samples consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with *L. intracellularis* purified from cell culture.

Quantitative PCR Analysis. qPCR analysis was conducted on fecal samples as previously reported, including a general bacteriology 16 sRNA for quality control (Pusterla *et al.*, 2010; Sampieri *et al.*, 2013). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla *et al.*, 2008, 2009).

Elemental [Fe] and [Ga] tissue concentration

Serum and intestinal tissue (IT) concentrations of elemental gallium ([Ga]) and iron ([Fe]) were determined through inductively coupled plasma – mass spectrometry (ICP-MS). The maltol portion of the initial compound was not detectable due to its rapid metabolism; hence, the tissue analysis focused on elemental metal concentrations (Bernstein *et al.*, 2000). The methodology implied standards comparison, prior to each batch of sample testing with rabbit blank serum, standard samples spiked with known [Ga] and [Fe], and verified on a batch of test tissues (liver). If the difference was >10% outside the range of comparison, the results were rejected and the analysis was repeated. Comparison between standards often implies a coefficient of variation, which differs for each metal. Analysis was always repeated in duplicate.

Pharmacokinetic analysis

Serum [Ga], or C_s, vs. time data were analyzed using the noncompartmental approach (NCA), and the analysis was carried out using GraphPad Prism 5.4 Software (GraphPad Software, Inc., La Jolla, CA, USA). The NCA was used to provide robust estimates of area under the curve from time 0 to time 216 h ($AUC_{0\to216}$), area under the curve extrapolated to infinity ($AUC_{0\to\infty}$), elimination rate constant (λ), mean residence time (MRT), terminal half-life ($t_{1/2}$), and oral clearance (Cl/F). The observed maximum plasma concentration (C_{max}) and observed time to maximum concentration (t_{max}) were determined by visual inspection of the serum concentration vs. time profiles.

The AUC was determined using the trapezoidal rule with the extrapolated area calculated from the ratio of the last calculated concentration and λ. Oral clearance (Cl/F) was calculated from the ratio of oral dose and AUC extrapolated to infinity ($AUC_{0\to\infty}$); MRT was calculated as the ratio of the area under the first moment curve ($AUMC_{0\to\infty}$) to the $AUC_{0\to\infty}$; and half-life was obtained from the product of 0.693 and MRT.

Statistics

Descriptive statistics (average and standard deviation, among others) were calculated for each NCA-derived parameter. For comparison of the PK parameters, a Student *t*-test was used. For the [Ga] and [Fe] in intestinal tissues, a one-way ANOVA was used; whereas a two-way ANOVA for repeated measures was used for PCR comparisons in relation to GaM treatment. For both ANOVAS, a Bonferroni *post hoc* test was implemented. When it was more appropriate not to assume a Gaussian distribution (uneven groups), a Kruskal–Wallis analysis with Dunn's *post hoc* test was used. Alpha was set at 5% for all the comparisons.

RESULTS

Clinical appearance

All rabbits tolerated well the single oral GaM dose. During the 24 h pilot study, no abnormalities were detected in either uninfected control or EPE-infected rabbits. In the pharmacokinetic studies, no change was evident in either group, for the duration of the experiments for appetite, fecal consistency, self or mutual grooming, and behavior. Only a marginal weight loss was observed on day 1 PT due to blood sampling loss and stress associated with multiple blood sampling (nine samples in 12 h). In all cases, BW rapidly recovered within a day (Fig. 1a,b). A decrease in BW gain (indicating a clear weight loss, or a BW gain smaller than half the average daily BW gain observed throughout the experiment) was observed in three EPE-infected rabbits (Fig. 1b) on or around 11 days PI, although it was not statistically significant ($P = 0.97$) overall.

Pharmacokinetics

Gallium was rapidly absorbed, with a minimal to nonexistent lag phase (Fig. 2a,b). The mean of the first detected C_s immediately after nasogastric dose was 57.3 μg/L in controls and 110.2 μg/L in EPE-infected rabbits, increasing to 389.5 μg/L and to 533.75 μg/L, respectively, within 30 min. In control does, C_{max} ranged between 248 μg/L and 849.5 μg/L [mean 500.43 ± 214.07 (SD) μg/L], and it peaked with t_{max} between 1–12 h (mean 3.41 ± 4.22 h). In EPE-infected rabbits, C_{max} ranged between 238 and 1415 μg/L (590.7 ± 416.07 μg/L), and t_{max} had a range of 0.5–1.5 h (0.9 ± 0.37 h).

Elemental gallium half-life in rabbits (uninfected controls and EPE-infected alike) is prolonged, particularly in the EPE-infected rabbits. The elimination rate constants, λ, were
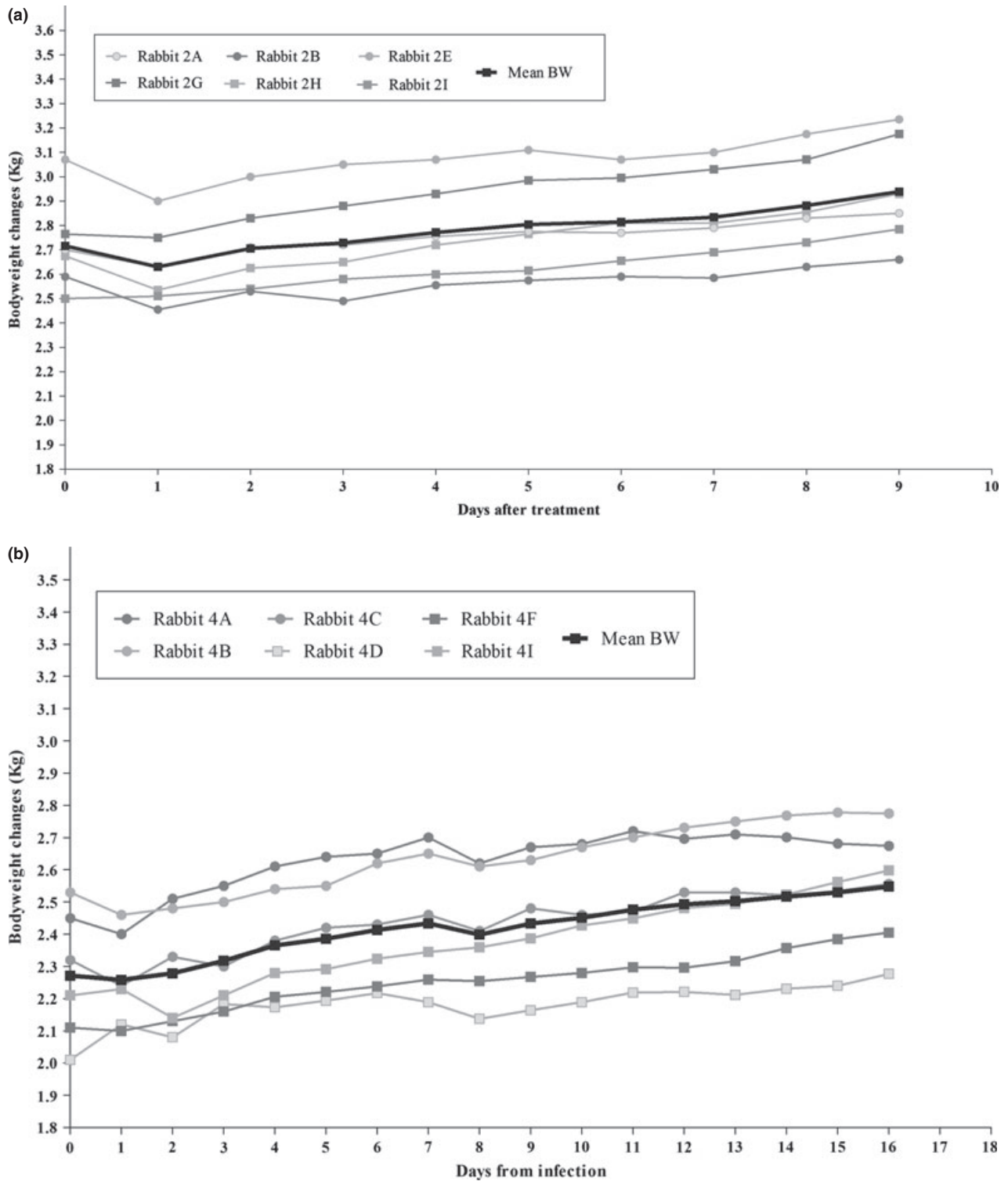
Fig. 1. (a) Body weight (BW, in Kg) changes in six uninfected control rabbits (PK study only) administered a single oral dose of gallium maltolate (50 mg/Kg) on day 0, with serial blood sampling until day 9 post-treatment (PT). Please note the bold line indicating the mean daily BW gain; BW loss on day 1 PT is ascribed to the stress of dosing and blood sampling on day 1 PT. (b) Body weight (BW, in Kg) changes in 6 EPE-infected rabbits (PK study only) inoculated with *Lawsonia intracellularis* on day 0 and administered a single oral dose of gallium maltolate (50 mg/Kg) on day 7 post infection (PI). The bold line indicates the mean daily BW gain; rabbits underwent blood sampling until day 9 PT or 16 PI. Note that the BW loss on day 8 PT is ascribed to the stress of dosing and serial blood sampling, but a trend toward BW gain suppression is noted around day 12 PI and is consistent with EPE infection.
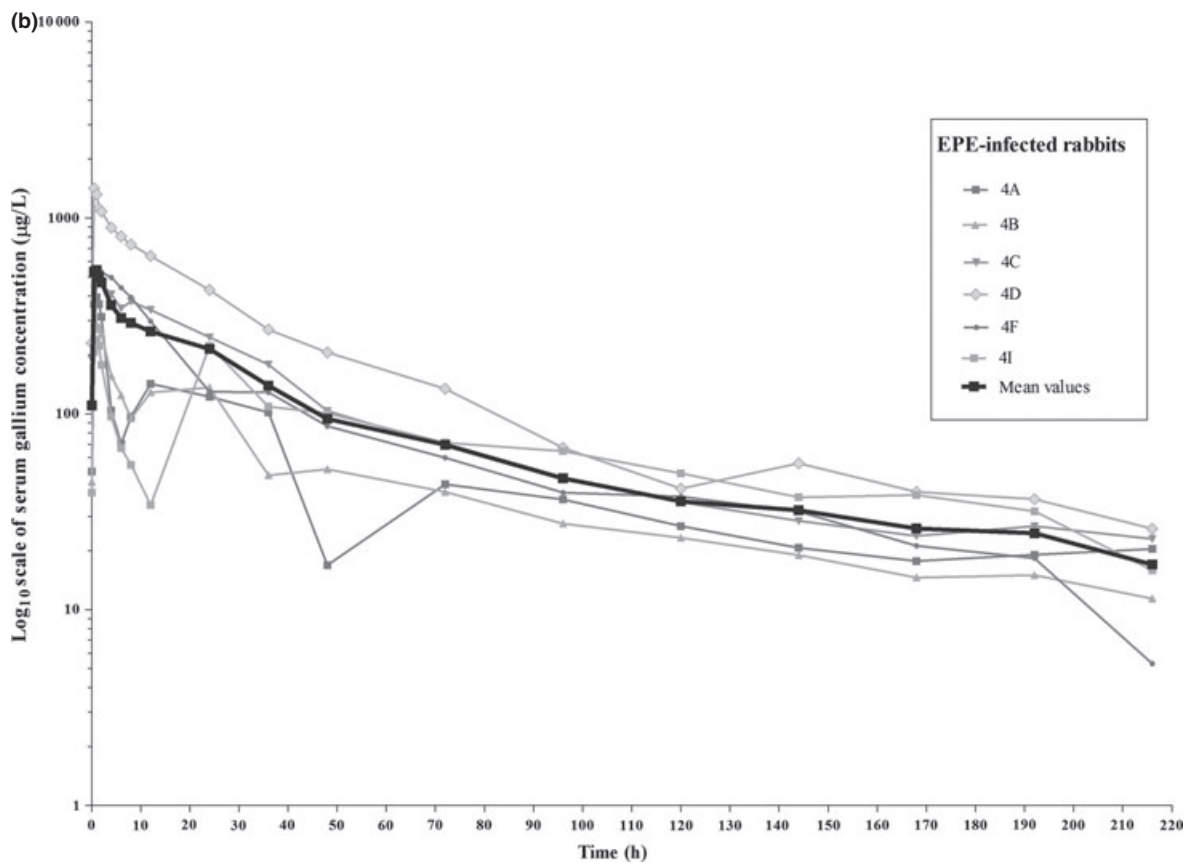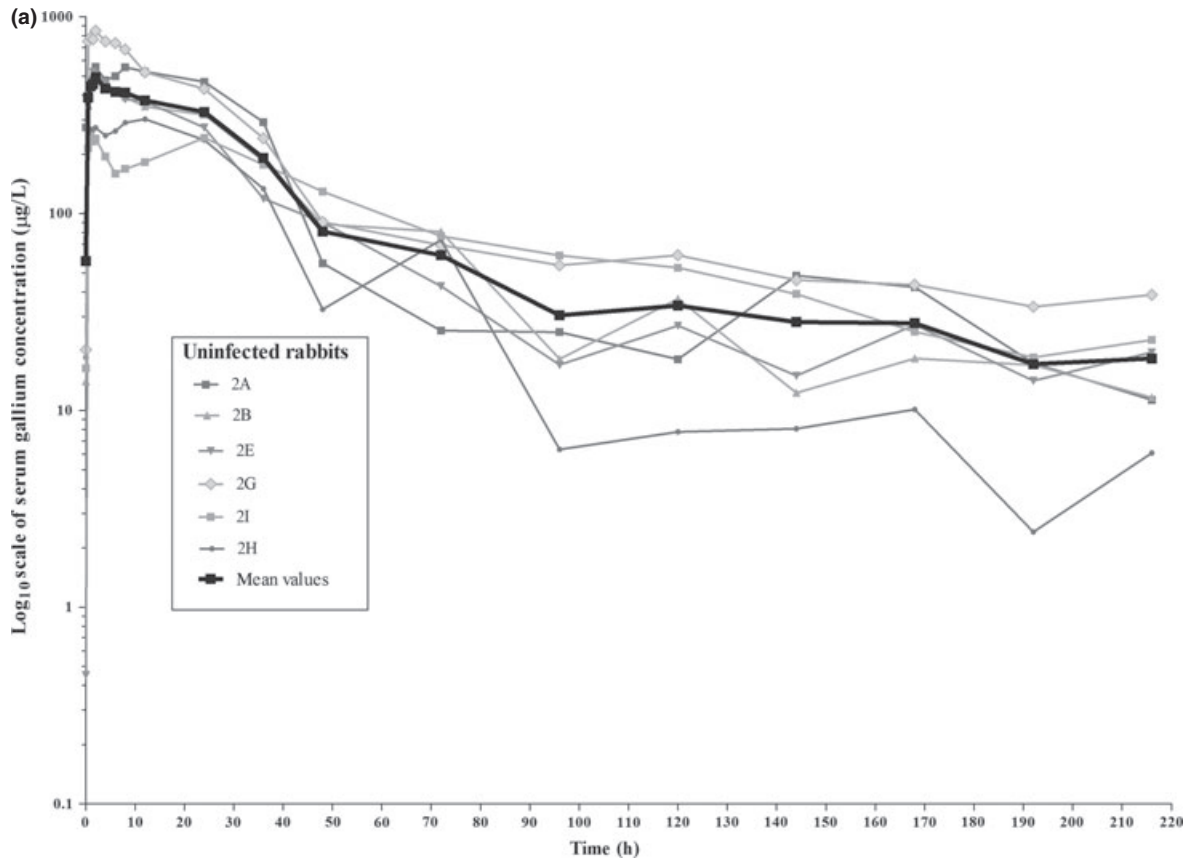
(a)

(b)

Fig. 2. (a) Representation on log-linear scale of gallium serum concentrations in six uninfected rabbits, treated orally once with gallium maltolate (50 mg/kg). Blood sampling lasted for 9 days (216 h) PT. Please note the bold line indicating the mean values. (b) Representation on log-linear scale of gallium serum concentrations in six rabbits infected with *Lawsonia intracellularis* and treated orally once with gallium maltolate (50 mg/kg). Blood sampling lasted for 9 days (216 h) PT. Please note the bold line indicating the mean values. Also, in both graphs note that in the first 24 h cecotrophy was not completely prevented in all rabbits; hence, a second peak in the curves is noticeable.

0.0171 ± 0.0028 per hour and 0.0116 ± 0.004 per hour, elimination half-lives of 39.38 ± 10.78 h and 59.36 ± 24.02 h; and oral clearance of 6.743 ± 1.887 L/h and 7.208 ± 2.565 L/h, respectively, in uninfected controls and EPE-infected rabbits. In both groups, the slope calculations were verified through the fitting of the line calculations and the evaluation of residuals, which showed a random distribution and close proximity to the fitted line.

The total body exposure to the drug showed a mean AUC extrapolated to infinity ($AUC_{0 \to \infty}$) of 21069.47 ± 6263.01 [(μg/L) × h] and 19146.35 ± 9403.13 [(μg/L) × h], respectively, in uninfected controls and EPE-infected rabbits. Averaged and detailed C_s values from each rabbit are reported in Table 1 for uninfected controls, and Table 2 for EPE-infected rabbits. The only statistically significant parameter between the healthy and EPE-infected rabbits was the elimination rate constant λ ($P = 0.03$), whereas no parameter differences were found within the two groups, as indicated in Table 2. Individual log-linear C_s vs. time curves are represented in Fig. 2a,b for healthy and EPE-infected rabbits, respectively. In several cases, cecotrophy ingestion, typical of normally behaving rabbits, could not be prevented, despite close monitoring, and it is clearly visible in the figures.

Our estimates of the $AUC_{0 \to \infty}$ were considered reliable; in the control group, the tail region estimate was always below 20% and in three rabbits was below 5%, whereas in the EPE-infected group, all but one doe (4A) had a tail region estimate below 20%, and two does (4D and 4F) were below 5%.

Gross pathology

In the limit of detection pilot study, rabbits were euthanized 1 day PT (8 days PI in EPE-infected rabbits). In the PK study, euthanasia was performed 9 days PT (16 days PI in EPE-infected rabbits), after collection of the last blood sample. No abnormalities were observed in the gastrointestinal tract (GIT) and in any other organ in the uninfected control rabbits. The EPE-infected rabbits euthanized on 1 day PT presented serosal edema (three rabbits), hyperplasia (three rabbits) and mild-to-moderate distention/fluid content of the mid-jejunum (two rabbits) compared with the rest of the small intestine. In the GIT of EPE-infected rabbits of the PK study lesions consistent with *L. intracellularis* infection were noted, such as hyperplasia, congestion, and corrugation of the mucosa of jejunum (six rabbits), ileum (two rabbits), and ileocecocolic valve (three rabbits), along with mild-to-moderate thickening of the serosa on the antimesenteric aspect of jejunum (six rabbits) (Sampieri *et al.*, 2013). No other gross abnormalities were detected in any other organ.

Intestinal tissue concentration

A difference of three orders of magnitude between [Fe] and [Ga] was detected in intestinal tissues, with [Fe] detected in ppm (mg/kg) and [Ga] in ppb (μg/kg). In healthy rabbits euthanized 1 day PT (pilot study), no significant difference was observed between the concentrations in the six different GIT sections collected for [Ga] ($P = 0.26$) or [Fe] ($P = 0.58$). In uninfected control rabbits, euthanized 9 days PT, a significant difference in [Ga] and [Fe] ($P < 0.0001$, for both) was observed between the six different GIT regions collected. For [Ga], the greatest values were determined in the cecal appendix [mean: 228.4 ± 61.3 (SD) μg/kg] and lowest in mid-jejunum (103.9 ± 39.6 μg/kg), cecum (107.7 ± 53.2 μg/kg), and colon (60.7 ± 18.4 μg/kg). Similarly for [Fe], the greatest concentration was found in the cecal appendix (11.3 ± 3.8 mg/kg). A comparison between uninfected control rabbits sacrificed 1 day PT vs. 9 days PT indicates a significantly lower [Ga] and [Fe] in GIT sections of subjects euthanized 9 days PT ($P < 0.0001$ and $P = 0.0011$, respectively) with the comparisons between the two sets of *ceca* and *cola* showing the greater differences for [Ga], but no specific differences for [Fe].

Among the EPE-infected rabbits euthanized 1 day PT, significant differences were observed between the six different GIT sections collected for [Ga] ($P = 0.031$) and [Fe] ($P = 0.015$), with greater concentrations in cecum and colon, and lower concentrations in the cecal appendix for both metals (Fig. 3a,b, respectively for gallium and iron). Among the EPE-infected rabbits euthanized 9 days PT, a significant difference in [Ga] and [Fe] ($P < 0.0001$, for both) was observed between the six different GIT regions collected, with the greatest concentration in the cecal appendix (160.9 ± 27.6 μg/kg) and the lowest in the colon (37.1 ± 7.4 μg/kg) for [Ga], but the highest in the cecum (40.9 ± 9.5 mg/kg) and lowest in the cecal appendix (9.7 ± 2.2 mg/kg) for [Fe]. In the comparison of the uninfected and EPE-infected rabbits, euthanized 9 days PT, [Ga] and [Fe] were significantly different in the two groups ($P < 0.0001$, for both) with the greatest differences observed in the end-jejunum, ileum, and cecal appendix for [Ga] (Fig. 4a), but only in the cecum for [Fe] (Fig. 4b).

Serology

The uninfected control rabbits' serology was negative for specific anti-*L. intracellularis* antibodies. For EPE-infected rabbits, titers were negative at day 7 PI, started rising at day 14 PI, and increased even further by day 16 PI (9 days PT, and time of euthanasia), confirming the presence of infection and reflecting data previously published (Sampieri *et al.*, 2013).

Table 1. Uninfected control rabbits

PK parameters (units)	2A	2B	2E	2G	2H	2I	Mean	SD
C_{max} (µg/L)	557	519	525	849	302	248	500.4	214
t_{max} (h)	2	2	1.5	2	12	1	1.75	0.41
λ (per h)	0.0168	0.0188	0.0178	0.0155	0.021	0.0127	0.0171	0.0028
$AUC_{(0\rightarrow\infty)}$ (µg·h/L)	24419.2	19816.1	18318.4	31480.9	13047.0	19335.0	21069.4	6263
$AUMC_{(0\rightarrow\infty)}$ (µg·h²/L)	117 0142	979 910	103 3179	209 8963	532 047	161 6948	123 8532	547 063
Cl/F (L/h)	5.52	6.43	8.24	4.05	9.92	6.3	6.743	1.887
MRT (h)	47	49	56	66	40	83	56.83	15.56
$t_{1/2}$ (h)	32.5	33.9	38.8	45.7	27.7	57.5	39.3	10.7

Table 2. Pharmacokinetic parameter estimates for 6 EPE-infected rabbits after a single oral bolus dose of gallium maltolate (50 mg/kg BW). The individual values, mean and SD are reported. In the last column the *P*-value derived from *t*-test comparisons between uninfected controls and EPE-infected rabbits are reported indicating the level of significance. Significantly different parameters are shaded in gray in the table

| PK parameter (units)	4A	4B	4C	4D	4F	4I	Mean	SD	P-value
C_{max} (µg/L)	393	468	477	1415	549	238	590.4	417	0.45
t_{max} (h)	1	0.5	1.5	0.5	1	1	0.9	0.37	0.20
λ (per hour)	0.0059	0.0087	0.0139	0.0168	0.014	0.0105	0.0116	0.004	0.03
$AUC_{(0\rightarrow\infty)}$ (µg·h/L)	13382.6	10664.3	20949.6	36990.6	17023.5	15867.2	19146.3	9403	0.53
$AUMC_{(0\rightarrow\infty)}$ (µg·h²/L)	1965962	980040	1467763	2039778	922321	1564630	1490082	472689	0.13
Cl/F (L/h)	10.08	10.42	5.87	2.88	6.6	7.4	7.208	2.565	0.74
MRT (h)	146	91	70	55	54	98	85.66	34.66	0.12
$t_{1/2}$ (h)	101.1	63	48.5	38.1	37.4	67.9	59.36	24.02	0.12

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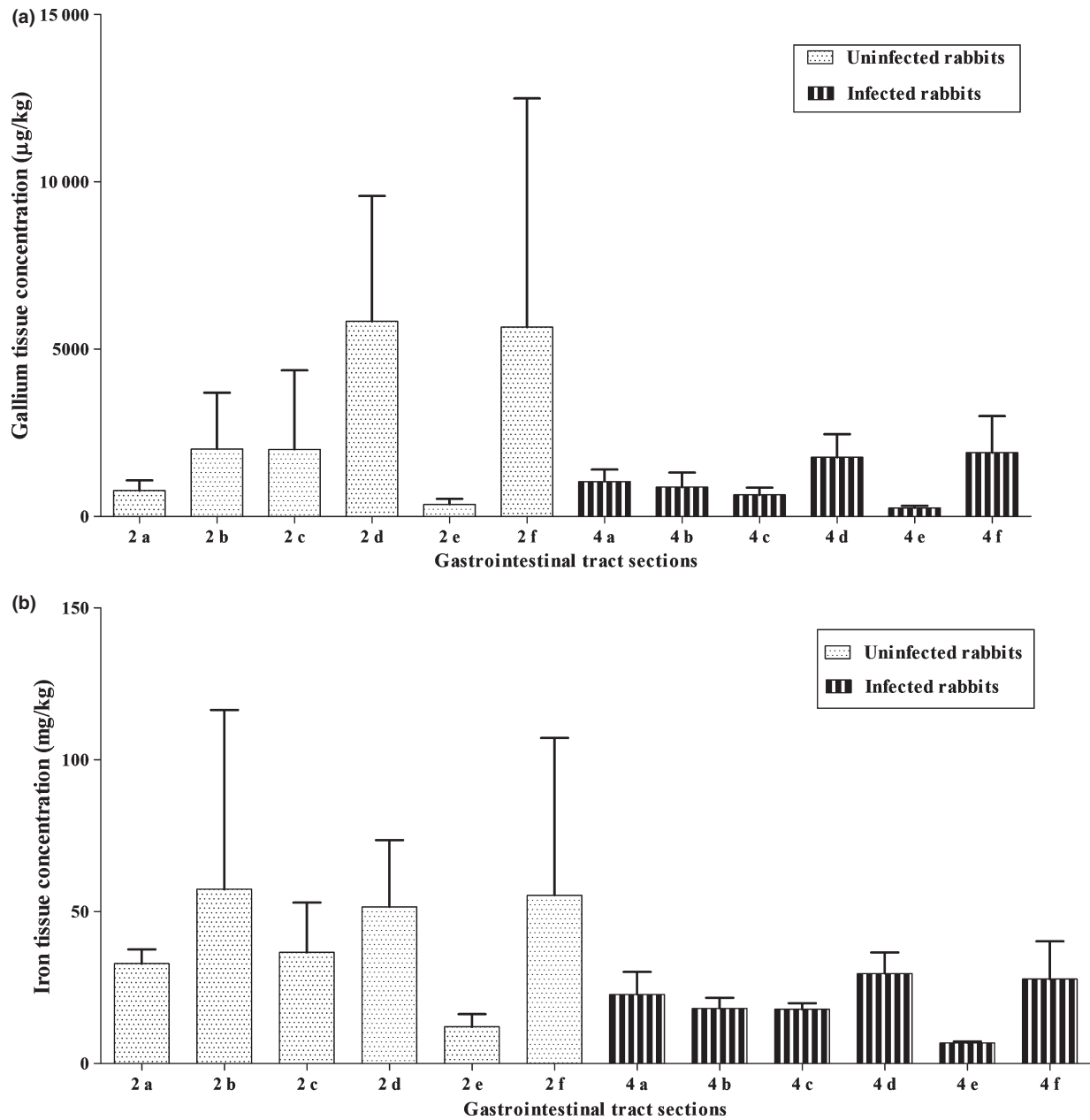
Fig. 3. (a) Comparison of [Ga] (in μg/kg) in sections of the small and large intestine of three uninfected controls and 3 EPE-infected rabbits, treated with a single oral dose of gallium maltolate (50 mg/kg) and sacrificed 1 day PT (8 days PI, for the EPE-infected subjects). (b) Comparison of [Fe] (in mg/kg) in sections of the small and large intestine of three uninfected controls and 3 EPE-infected rabbits, treated with a single oral dose of gallium maltolate (50 mg/kg) and sacrificed 1 day PT (8 days PI, for the EPE-infected subjects). In both (a) and (b) sections, each bar graph represents the mean value (with whiskers indicating the SD) of each intestinal section ($n = 3$): a = mid-jejunum; b = end-jejunum; c = ileum; d = cecum; e = cecal appendix; f = colon.

Histology and immunohistochemistry

In uninfected controls, no alterations of the intestinal structure were noted, and no IHC labeling with specific murine anti-*L. intracellularis* antibodies was detected, in the prime areas usually infected by *L. intracellularis* (jejunum, ileum, and cecum). In EPE-infected rabbits, the IHC labeling confirmed typical lesions in seven of nine animals (total). Furthermore, without bacterial inoculation, no infection occurs ($P = 0.002$),

with an odds ratio of developing infection 57 times higher after exposure to *L. intracellularis*. In the EPE-treated group, no statistical difference was detected with regard to the location of the lesion (jejunum, ileum, and cecum), regardless of the severity of the lesions (graded from 0 to 4, being 0 equal to no lesions and 4 equal to 100% lesions) (Guedes & Gebhart, 2003b); the frequency within one intestinal section vs. another; or the detection of lesions in more than one section in the same rabbit GIT ($P = 0.07$).
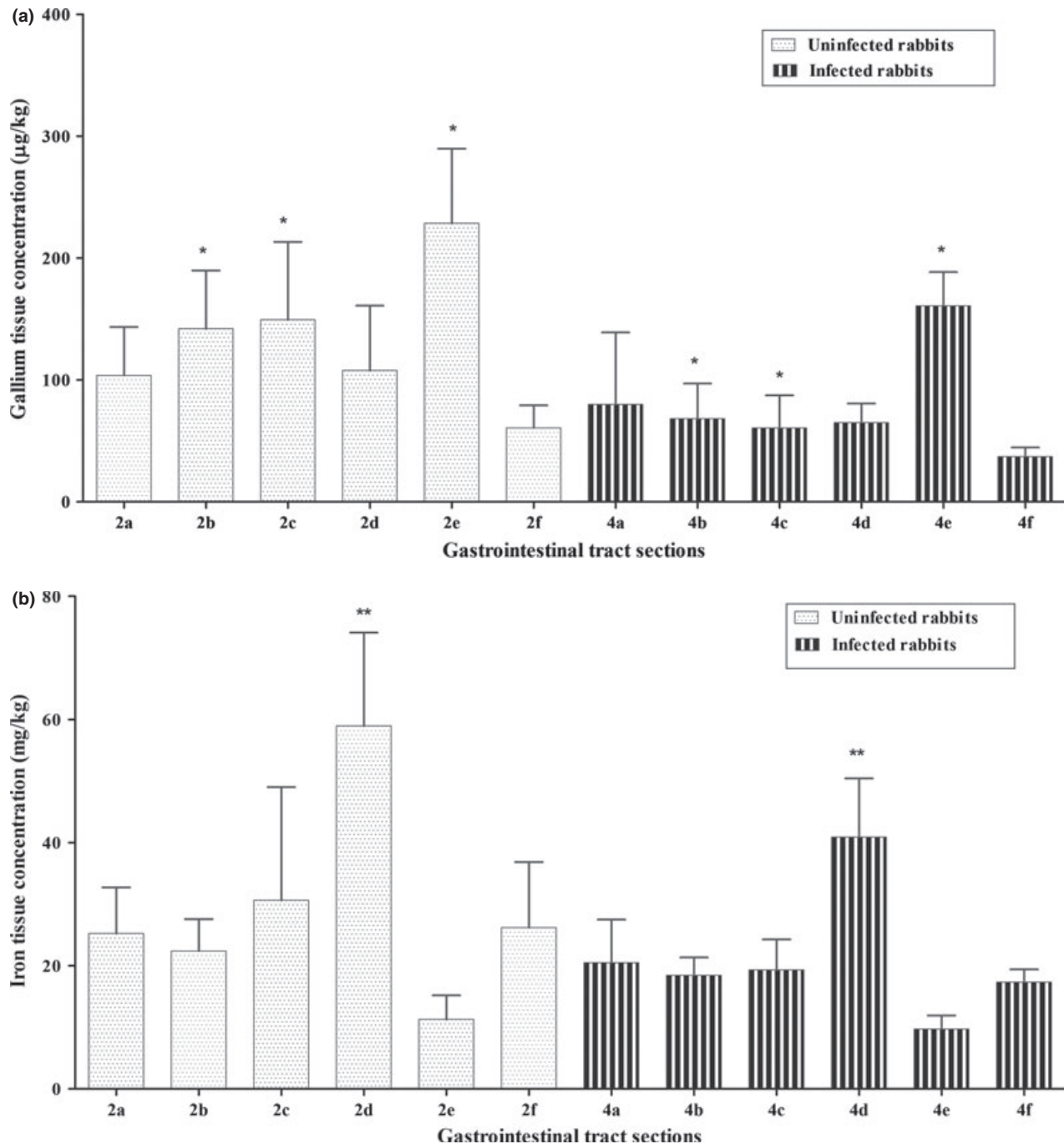
Fig. 4. (a) Comparison of intestinal tissue [Ga] (μg/kg) in six uninfected controls and 6 EPE-infected rabbits after a single oral gallium maltolate dose (50 mg/kg BW). Samples collected 9 days PT (or 16 days PI). (b) Comparison of intestinal tissue [Fe] (mg/kg) in six uninfected controls and 6 EPE-infected rabbits, after a single oral gallium maltolate dose (50 mg/kg BW). Samples collected 9 DPT (16 DPI). In both graphs, each bar graph represents the mean value (with whiskers indicating the SD) of each intestinal section ($n = 6$): a = mid-jejunum; b = end jejunum; c = ileum; d = cecum; e = cecal appendix; f = colon.

PCR

A complete absence of *L. intracellularis* shedding was confirmed in feces of uninfected control rabbits ($P < 0.0001$). In the EPE-infected rabbits, fecal shedding of *L. intracellularis* was confirmed (Fig. 5a,b) at around day 4 PI, in partial agreement with previous reports, as it started earlier than day 7 PI and lasted for the duration of the experiment (Sampieri *et al.*, 2013). No differences were noted with regard to comparisons between fecal shedding in rabbits ($P = 0.53$), between days ($P = 0.21$) or between *L. intracellularis* shedding in fecal and cecal content (at necropsy, $P = 0.7$).
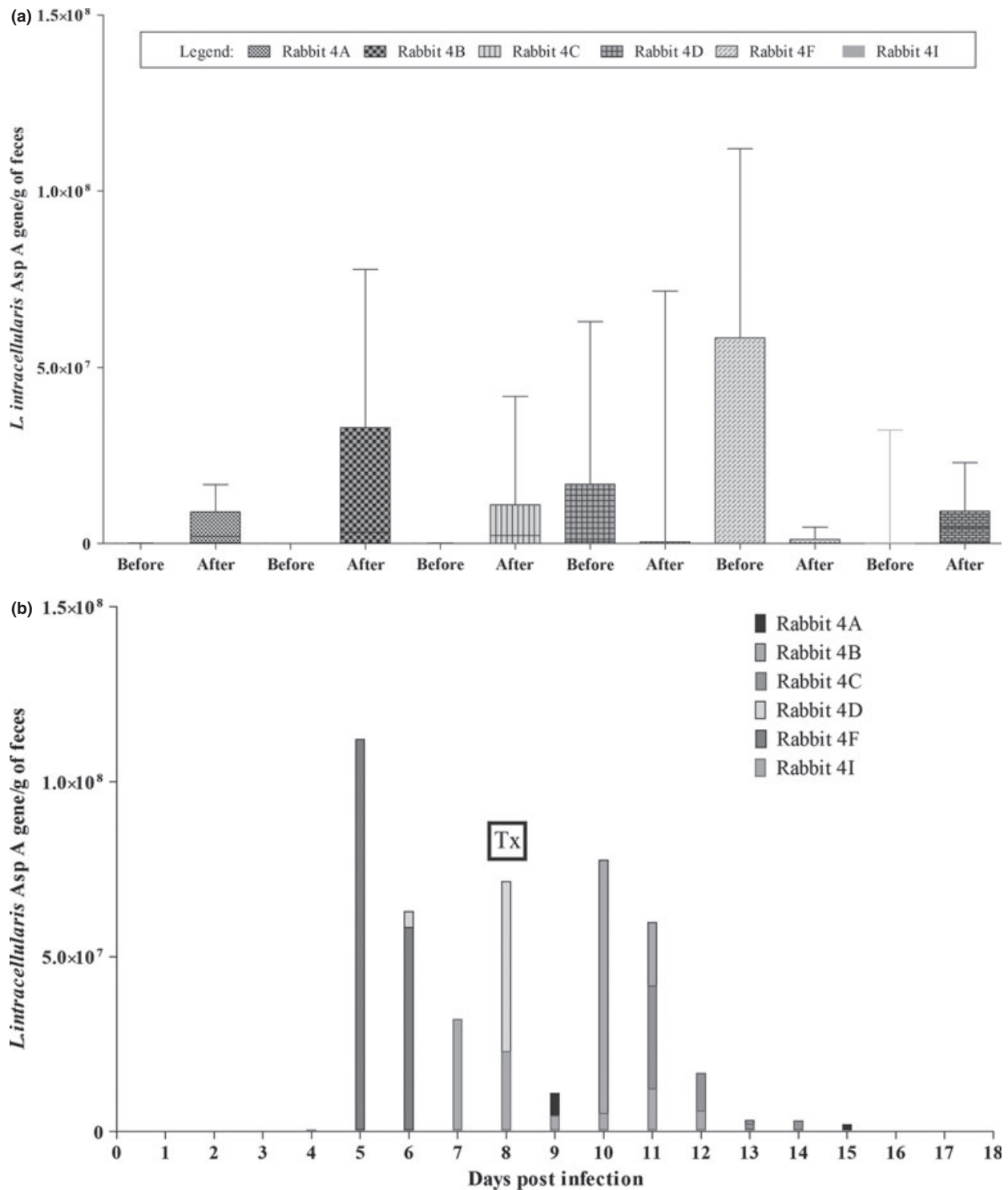
Fig. 5. (a) *Lawsonia intracellularis* DNA (*AspA* gene)/gram of feces following *L. intracellularis* qPCR analysis in feces of experimentally infected rabbits before and after a single oral dose of gallium maltolate (50 mg/kg). Each pair of bars represents an individual rabbit before and after treatment. (b) *L. intracellularis* DNA (AspA gene)/gram of feces following *L. intracellularis* qPCR analysis in feces of experimentally infected rabbits treated with a single oral dose of gallium maltolate (50 mg/kg). Superimposed bars represent each individual rabbit concentration and 'Tx' indicates the day of treatment.

DISCUSSION

In this study, a single oral bolus dose of GaM was readily tolerated by uninfected and EPE-infected rabbits. However, EPE infection caused significant differences in the estimates of λ and consequently a prolonged terminal half-life ($t_{1/2}$), even though no significant difference was found between the two groups, along with other estimated PK characteristics. Given

the long half-life, a GaM dosing interval of once every 48 h should lead to sufficient concentrations in rabbits for AM activity of GaM. This dosing interval was deemed safe to test in a efficacy study, although the potential for elemental [Ga] accumulation and environmental impact requires further consideration (Fecteau *et al.*, 2011).

The *in vitro* susceptibility studies for *L. intracellularis* clarified that multiple AM dosing is necessary for therapy, due to the variable distribution and differentiation state of the infected cells in the enteric crypts (Wattanaphansak *et al.*, 2009a). Furthermore, no clinical study has shown efficacious AM therapy against EPE following a single dose or short duration AM dosage regimen (Lavoie *et al.*, 2000; Sampieri *et al.*, 2006; Frazer, 2008). The 48 h dosing interval was derived from estimates for the elimination half-life in EPE-infected rabbits, as half-life is the only factor determining the time to steady-state concentrations. As well, assuming linear pharmacokinetics and according to the principle of superposition, our single GaM dose's AUC estimate suggests that the 48 h dosing interval is adequate to assure appropriate exposure for AM therapy. These assumptions are necessary as multiple dosing studies of gallium-based compounds for PK purposes are not common in animals, with only one study for GaM reported in foals (Martens *et al.*, 2010) and a derived study on chemoprophylactic activity derived from it (Chaffin *et al.*, 2011), and a multiple dosage regimen estimated for gallium nitrate in adult horses based on a projected study *in silico* (Pollina *et al.*, 2012). We did not administer an intravenous dose to rabbits, which precluded the calculation of absolute GaM's bioavailability, but in healthy humans, GaM is reported to provide a GaIII bioavailability of ≥25–57% and exhibits linear absorption and elimination kinetics (Bernstein *et al.*, 2000). Presuming similar bioavailability of GaM in rabbits, such data also supported our decision of a 48 h dosing interval for GaM in EPE-infected rabbits.

Our study using the EPE rabbit model is intended to be a preclinical study to help in determining the clinical GaM treatment of foals affected by this illness. An effective GaM therapy against *L. intracellularis* in an infection rabbit model of EPE might allow allometric scaling of a GaM dose for a clinical trial in weanling foals. Also, despite the anatomical similarities between rabbits and horses (e.g., herbivores, hind-gut fermenters with a small stomach, and large cecum), we would still expect to observe significant interspecies variation and hence, the need to conduct dose titration studies in foals (Bernstein *et al.*, 2000). Certainly, the design of our rabbit study using a 9-day sampling period was premised on information available in humans ($t_{1/2} \approx 5$ days), rather than foals ($t_{1/2} \approx 1.5$ days), and a consideration of body-scaling in rabbits, along with putative effect of EPE infection on GaM absorption and disposition characteristics (Bernstein *et al.*, 2000; Martens *et al.*, 2007).

It is relevant to compare our findings to what Martens *et al.* (2007) estimated in healthy neonatal foals, where MRT was 39.5 ± 17.2 h, and $t_{1/2}$ was 26.6 ± 11.6 h (Martens *et al.*, 2007). The MRT and $t_{1/2}$ in juvenile rabbits were much longer. Interestingly, in 2010, the same researchers reported several

differences in the PK parameters estimates of GaM administered to adult horses (Arnold *et al.*, 2010). Sampling lasted 5 days, and the main PK estimates were generally more fitting with the rabbits' PK findings, being C_{max}: 280 ± 90 μg/L, t_{max}: 3.09 ± 0.43 h, lag-time: 0.26 ± 0.11 h, and apparent elimination $t_{1/2}$: a 48.82 ± 5.63 h (Arnold *et al.*, 2010). The differences in half-lives may relate to ontogenic differences in the degree of maturation of the drug clearance mechanisms between the neonate foal and the adult horse. Usually, drug clearance mechanisms are underdeveloped in neonatal stages resulting in longer half-lives relative to the adult. The study in foals reported by Martens and coworkers utilized a 48-h blood sampling period, which may have been insufficient to adequately describe the terminal disposition rate constant, on the concentration vs. time profiles of the foals in that study (Martens *et al.*, 2007). Furthermore, our juvenile rabbits gave PK estimates compatible with results reported in mice treated with the same dose once daily for 10 days (Harrington *et al.*, 2006), but were nearly four times lower than estimates collected in dogs after a single dose (Bernstein *et al.*, 2000).

In addition to identifying an appropriate dosing interval for GaM in rabbits, we wished to understand the influence of EPE infection on the PK of GaM. The EPE rabbit model demonstrated clearly the impact of GIT disease on both serum and tissue [Ga] and [Fe] following a single dose of a metal-based compound. To mimic a natural infection in foals (Lavoie *et al.*, 2000; Sampieri *et al.*, 2006; Frazer, 2008), the delay between the onset of infection (inoculation) and treatment was purposefully designed in our preclinical rabbit model, and current diagnostics were utilized to confirm the status of disease. It was expected that a single GaM dose would not affect the fecal shedding of *L. intracellularis* DNA and the immune-response (Sampieri *et al.*, 2013); thus, serology, PCR analysis and IHC tests confirmed the EPE-infected status in rabbits. In particular, IHC showed that only two of six total EPE-infected does, in the PK study, had no detectable lesions around the considered 'infection peak' (around 14 days PI). The three EPE-infected rabbits euthanized at 8 days PI had confirmed IHC lesions too, despite subtler apparent changes (typical of the EPE rabbit model at that stage) of the serosal and mucosal layers of jejunum and ileum on gross pathology (Sampieri *et al.*, 2013). Thus, despite one GaM treatment, our results underlined that both classic macroscopic and immunohistochemical EPE lesions were found.

It is difficult to determine whether the apparently faster (day 16 PI) cell turnover/healing (usually noted at 20–21 days PI, in the original infection model) was due to the natural evolution of the lesions (Sampieri *et al.*, 2013), or GaM effects on macrophage activity, similar to the action of other gallium compounds on various cell-lines of the reticulo-endothelial system (Bockman, 1991). However, intracellular [Ga] in activated macrophages was not assessed in this lapine model, or in the equine experiments described thus far (Martens *et al.*, 2007; Arnold *et al.*, 2010). In the rabbit model, we assessed [Ga] and [Fe] in targeted GIT sections and not in macrophages for two reasons: to assess the impact of disease on the tissue

content of these oligo-elements and because EPE is uncommonly associated with large inflammatory reactions. In the equine PK studies, both foals and horses utilized were clinically normal (Martens *et al.*, 2007; Arnold *et al.*, 2010).

In uninfected control rabbits, a single oral GaM dose did not cause major differences in tissue accumulation within a day PT. However, in rabbits sacrificed 9 days PT, [Ga] and [Fe] in tissues tended to show reverse correspondence in specific GIT sections: gallium preferentially accumulated in the cecal appendix tissue, where reticulo-endothelial tissue is mostly concentrated, but this finding was true in reverse for [Fe]. Whether this phenomenon is dependent upon GaIII sequestration by macrophages is not known at this time.

Not surprisingly, EPE infection led to decreased tissue [Ga], particularly where lesions were more pronounced (i.e., terminal jejunum and ileum), as the intestinal absorption is severely impaired (Wong *et al.*, 2009). Moreover, [Fe] was decreased throughout the entire GIT in the EPE-infected rabbits. Reductions in [Fe] may be due to competition with GaM for tissue uptake and disruption in iron absorption due potentially to saturation of FeIII transporters (e.g., transferrin and siderophores). It is important to note that [Fe] was decreased in the cecum, despite the prolonged residence of *digesta* in that GIT section, and a single GaM dose can alter both iron absorption and catabolism equilibria, despite the normal iron dietary levels.

In conclusion, our study allowed the first evaluation of GaM PK in uninfected and in EPE-infected rabbits, along with an assessment of iron tissue contents achieved after a single GaM dose. A dosing interval of *q* 48 h was estimated to sufficiently maintain therapeutic concentrations during EPE infection in rabbits. Nonetheless, dose titration studies and assessment of safety of such a dosage regimen (50 mg/kg *q* 48 h) should be carried out in foals, before employing this therapy in clinical patients. Finally, it should be considered that EPE-related malabsorption may result in decreased GIT tissue [Ga] and [Fe] in EPE-infected animals. Thus, the use of GaM as AM alternative therapy warrants further assessment, not only for its ability to compete with the essential nutrient iron, but also for its impact on recovery from infection.

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