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RESEARCH ARTICLE

Effect of Probiotics on the Pharmacokinetic Aspects and Tissue Residues of Difloxacin in Broiler Chickens

Mohamed Hend F[1], Atta AH[2]*, Darwish AS[1] and Atef M[2]

[1]Department of Biochemistry, Animal Health Research Institute, Dokki, Giza, Egypt
[2]Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt
*Corresponding author: attaattia52@yahoo.com

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A B S T R A C T

The present work was carried out to test the impact of oral probiotics on the pharmacokinetics of difloxacin (IV and oral; 10mg/kg b.wt) and its tissue residues in broiler chickens. Blood samples were taken over 24 h of difloxacin administration for the pharmacokinetic study. For testing difloxacin residues, the drug was administered orally at the same dose for 5 successive days and the edible tissues (muscle, liver, kidney and gizzard) were collected from slaughtered birds after 1, 3, 5 and 7 days of the last dose. The drug was estimated by using high-performance liquid chromatography (HPLC). The two-compartment open model was applied to describe the plasma concentration-time data of difloxacin. Analysis of the data following intravenous (IV) administration revealed a significantly higher plasma concentration of difloxacin (0.16±0.0 *vs* 0.13±0.0 µg/ml at 24 h post injection; $P<0.05$) and a significantly prolonged half-life (4.09±0.03 *vs* 3.75±0.02 h) in probiotic-pretreated chickens as compared to non-treated one. The absorption half-life ($t_{0.5ab}$) of difloxacin was 1.34±0.03 and 1.43±0.04 h ($P<0.05$) and the calculated oral bioavailability (F) was 72.82±5.35% and 64.56±5.29% in chickens with and without probiotic pretreatment, respectively. The elimination half-life of difloxacin was more prolonged ($P<0.05$) in probiotic pretreated chickens. The residues levels in muscle, liver and kidney were lower in probiotic-pretreated chickens. Twenty-four hours after the last oral dose, the tissue residues of difloxacin were lower than the recommended MRLs in chicken. In conclusion, the use of probiotic modulate pharmacokinetics and tissue residue of difloxacin and increase its bioavailability.

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INTRODUCTION

The role of gut microflora and gut metabolizing enzymes in absorption, bioactivation, and the overall efficacy of orally administered drugs has become more profound (Stojančević *et al*., 2013). Animal's and human's gut contains trillions of bacteria cells that represent a complex ecosystem and produce a huge number of chemical molecules that can affect the host. The bacterial population present in the intestine participates in nutrition and metabolic processes of xenobiotics in the host. In this respect, there is increasing evidence on the impact of intestinal microflora on human and animal health (Sommer and Ba¨ckhed, 2013). In recent years there has been great interest in exploring the function and the mechanism of action of the gut microbiota. This raises ideas to select and propagate suitable bacterial strains that could be beneficial to the health and function of the intestinal mucosal barrier, improve its immune defense mechanism and strengthen its anti-inflammatory responses (Meijerink *et al*., 2013). Many probiotic strains such as *Lactobacillus*, *Streptococcus*, and *Bacillus* spp., as well as Bifidobacterium that are commonly used in feeding poultry, resulted in greater weight gain, higher feed conversion, and decreased morbidity and mortality (Santini *et al*., 2010). However, the uncontrolled use of probiotics may lead to several undesired side-effects (Guarner and Schlaafsma, 1998). Since the intestinal microflora plays such an important role in nutritional,

metabolic and immunologic processes, there is currently an interest in the effect of administered probiotics on its function and activity (Stojančević *et al*., 2013). However, information on the effect of probiotics on the disposition kinetics of orally given drugs is still very limited (Stojančević *et al*., 2013; Matuskova *et al*., 2014). The effect of probiotics on the disposition of antimicrobial agents is not yet well studied, although some knowledge on the simultaneous use of probiotics with antibiotics exists (Pavlova *et al*., 2015). Thus, a question arises whether the use of probiotics is safe when given simultaneously with an antibiotic. Therefore, the impact of probiotic on the pharmacokinetic profile of difloxacin was studied.

MATERIALS AND METHODS

Drugs and reagents: Difloxacin purity, 99.9% was purchased from Swede, Egypt. Probiotic composed of *Bacillus subtilis* 1×10^{11} CFU and *lactobacillus acidophilus* 1×10^{8} CFU was obtained from Naphavit, company, Vietnam. All chemicals were either of HPLC grade, of 99.9% purity or analytical grade.

Birds: One-day old chicks obtained from Elarabia poultry breeding farm, Egypt, were used. Chicks were reared under strict hygienic condition (20˚C room temperature; 55-60% relative humidity) and 14 hours of daylight. They were offered an antibiotic-free standard ration and allowed free access to fresh clean water. Chickens were handled according to the guidelines of the Institutional Animal Care and Use Committee, Veterinary Medicine, Cairo University (#: Vet CU20022020151).

Pharmacokinetic study: Twenty chicks were allocated randomly into four equal groups; two groups were fed normal feed and two groups were fed normal feed supplemented with probiotic from day 5 to day 21 via feed at a dose of 1g of probiotic /kg feed. At the 21st day, 2 hours after the last probiotic dose, difloxacin was injected intravenously in a single dose of 10 mg/kg into the right-wing vein of two groups of chickens (difloxacin and difloxacin + probiotic). For oral administration, difloxacin was administered in the same dose into the chickens of the other two groups (difloxacin and difloxacin + probiotic) by direct gavage into the crop at the same dose and time.

Blood samples: Blood samples (0.5ml each) were collected from the left-wing vein of each chicken through a previously inserted cannula into heparinized tubes at 5, 10, 15, and 30 min. and 1, 2, 4, 6, 8, 10, 12 and 24 h after IV injection of difloxacin and at 15, and 30 min. and 1, 2, 4, 6, 8, 10, 12 and 24 h after oral administration. Plasma was collected after centrifugation of blood samples at 3100 rpm for 10 min. and stored at -20ºC until analyzed for difloxacin.

Drug residue study: Two groups of 20 chickens each; (difloxacin and difloxacin + probiotic) were used. Each bird was administrated orally with difloxacin, 2 hours after the last probiotic dose, in a dose of 10mg/kg by gastric gavage, once daily for five consecutive days. Five chickens from each group were slaughtered after 1, 3, 5 and 7 days of the last dose. After slaughter, samples from liver, kidney, gizzard and muscle were taken from each bird and were kept frozen (-20°C) until assayed for difloxacin.

Preparation of standard curves: Drug-free normal chicken plasma and tissues were spiked with 0.01, 0.1, 0.5, 1, 2 and 5µg difloxacin /ml or g. Difloxacin was extracted from plasma and from tissue samples as previously described (Gigosos *et al*., 2000; Fernández-Varón *et al*., 2006a). Difloxacin concentrations were estimated by HPLC according to Cazedey *et al*., (2014). HPLC system with Agilent series 1200 quaternary gradient pump, series 1200 auto sampler, series 1200 UV Vis detector, and eclipse XDB C18 column (5um, 4.6mm, 210mm) was used. The mobile phase (5% acetic acid: methanol; 70: 30 v/v, pH 2.5) was allowed to flow at rate of 1 mL/min and the injection volume was 20 µL. The column temperature was maintained at 25ºC.The pharmacokinetic parameters were calculated by PK-Solver; an add-in program for Microsoft Excel.

In vitro protein binding: The in vitro protein-binding percent was estimated as described by Craig and Suh, (1991). Various concentrations of difloxacin were prepared in antibiotic-free chicken's serum as well as in phosphate buffer (pH 6.2). The inhibition zones of the growth of the *Bacillus subtili*s inoculated into agar medium by the different concentrations of difloxacin were measured. The percentage of the protein-bound fraction was calculated as follows:

Protein binding % = (Zone of inhibition in the buffer - zone of inhibition in serum)/ Zone of inhibition in buffer X 100

Statistical analysis: All results were expressed as mean±SD. The significance between means was tested with the Student's t-test at a probability level at P>0.05.

RESULTS

Twenty four hours following a single IV injection of difloxacin (10mg /kg b.wt.) in broiler chickens without and with probiotic pre-treatment, the mean plasma drug concentration was 0.13±0 and 0.16±0 µg/ml, respectively (Fig.1). The plasma concentration-time data of difloxacin (10mg/kg b.wt) following IV injection in broiler chickens without and with probiotic pretreatment was best described by the two compartments, open model. Oral administration of probiotic before IV injection of difloxacin resulted in a significantly higher plasma difloxacin concentration up to the 24 h period of drug sampling (Fig. 1).

The pharmacokinetic analysis of the data following IV administration revealed a significantly higher plasma concentration of difloxacin at zero time (12.09±0.07 *vs* 11.82±0.1h) and a significantly prolonged half-life (4.09±0.03 *vs* 3.75±0.02 h) in probiotic-pretreated chickens as compared to non-treated birds (Table 1). The volume of distribution and the clearance rate although achieved higher values, however, the significant difference did not occur. The areas under curves (AUC_{0-t}, $AUC_{0-\infty}$, AUMC) and the MRT were much higher in probiotic-pretreated chickens (Table 1).

Table 1: Pharmacokinetic parameters of difloxacin in probiotic *non-treated* and probiotic-pretreated broilers after a single IV dose of 10 mg/kg b.wt. (mean±SD, n = 5)

Parameters	Units	Difloxacin	Difloxacin + Probiotic
A	µg/ml	8.28±0.05	8.53±0.04*
α	h^{-1}	4.93±0.08	4.96±0.02
B	µg/ml	3.55±0.05	3.57±0.03
β	h^{-1}	0.18±0.00	0.17±0.00*
k_{10}	h^{-1}	0.57±0.00	0.53±0.00*
k_{12}	h^{-1}	2.94±0.05	3.02±0.02*
k_{21}	h^{-1}	1.61±0.03	1.58±0.01
$t_{1/2\alpha}$	h	0.14±0.00	0.14±0.00
$t_{1/2\beta}$	h	3.75±0.02	4.09±0.03*
C^0	µg/ml	11.82±0.1	12.09±0.07*
V	(mg)/(µg/ml)	0.85±0.01	0.83±0.00*
CL	(mg)/(µg/ml)/h	0.48±0.00	0.44±0.00*
V2	(mg)/(µg/ml)	1.55±0.02	1.58±0.02
CL2	(mg)/(µg/ml)/h	2.49±0.03	2.50±0.02
AUC_{0-t}	µg/ml.h	20.64±0.21	22.40±0.15*
$AUC_{0-\infty}$	µg/ml.h	20.87±0.21	22.76±0.16*
AUMC	µg/ml.h^2	104.23±1.13	124.59±1.57*
MRT	h	4.99±0.02	5.47±0.05*
V_{ss}	mg/(µg/ml)	2.39±0.03	2.4±0.02

A and B; zero-time intercept of distribution and elimination phase, α and β; rate constants of distribution and elimination, k_{10}, k_{12}, k_{21}; rate constants of the first-order kinetic, C^0; plasma concentration at zero time, $t_{1/2\alpha}$ and $t_{1/2\beta}$; half-life of distribution and elimination, V and V2; volume of central and peripheral compartments, Cl; clearance, AUC_{0-t} and $AUC_{0-\infty}$; area under the curve extrapolated to last plasma concentration and to infinity, AUMC; area under the first moment curve, MRT; mean residence time, V_{ss}; volume of distribution,. * P≤0.05.

Table 2: Pharmacokinetic parameters of difloxacin in probiotic non-treated and probiotic-pretreated chickens after a single oral dose of 10 mg/kg b.wt. (mean±SD, n = 5).

Parameters	Units	Difloxacin	Difloxacin + Probiotic
A	µg/ml	7.32±0.92	9.68±.61*
α	h-1	0.27±0.01	0.29±0.002*
B	µg/ml	2.65±0.59	2.53±0.45
β	h-1	0.37±0.01	0.4±0.01*
K_{ab}	h-1	0.48±0.01	0.52±0.01*
$t_{0.5ab}$	h	1.43±0.04	1.34±0.03*
k_{10}	h-1	0.28±0.01	0.30±0.00*
k_{12}	h-1	0.004±0.001	0.003±0.001
k_{21}	h-1	0.36±0.01	0.38±0.01*
$t_{1/2\alpha}$	h	2.56±0.06	2.40±0.02*
$t_{1/2\beta}$	h	1.89±0.01	1.75±0.01*
V/F	(mg)/(µg/ml)	2.63±0.30	2.05±0.14*
CL/F	(mg)/(µg/ml)/h	0.74±0.07	0.61±0.04*
V_2/F	(mg)/(µg/ml)	0.03±0.01	0.02±0.004*
CL_2/F	(mg)/(µg/ml)/h	0.01±0.002	0.001±0.001*
T_{max}	h	2.66±0.06	2.5±0.03*
C_{max}	µg/ml	1.80±0.19	2.31±0.17*
AUC_{0-t}	µg/ml.h	13.50±1.24	16.32±1.13*
AUC_{0-inf}	µg/ml.h	13.54±1.24	16.35±1.13*
AUMC	µg/ml.h^2	76.24±5.99	86.61±5.85*
MRT	h	5.64±0.11	5.30±0.04*

A and B; zero-time intercept of distribution and elimination phase, α and β; rate constants of distribution and elimination, $K_{ab;}$ rate constant of absorption, k_{10}, k_{12}, k_{21}; rate constants of the first-order kinetic, $t_{1/2ab}$, $t_{1/2\alpha}$ and $t_{1/2\beta}$; half-life of absorption, distribution and elimination, V and V_2; volume of central and peripheral compartments, Cl; clearance, C_{max} and T_{max}; peak serum concentration and time to peak concentration, AUC_{0-t} and $AUC_{0-\infty}$; area under the curve extrapolated to last plasma concentration and to infinity, AUMC; area under the first moment curve, MRT; mean residence time, * P≤0.05.

Table 3: Protein binding % of difloxacin in chicken serum *in vitro*.

Concentration	Protein binding%	
	Normal chicken serum	Probiotic pretreated-chicken serum
12.5	10	7.96
6.25	12.1	10.8
3.125	9.03	8.75
1.56	12.7	8.28
0.78	9.7	9.38
Mean±SD	10.7±0.7	9.03±0.5*

*P≤0.05; compared to difloxacin only treated chickens.

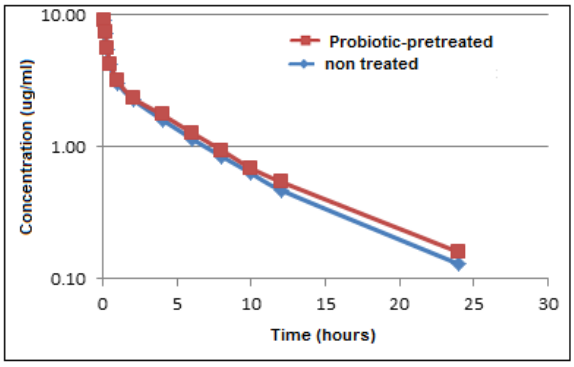

Fig. 1: Semilogarithmic graph showing the time-concentration of difloxacin in the plasma of *non-treated* **a**nd probiotic pre-treated chickens after a single IV injection of 10mg/kg.

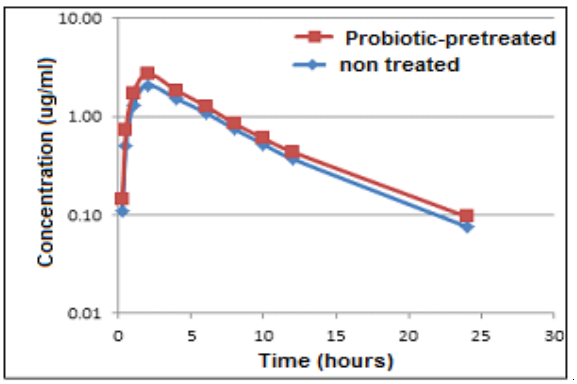

Fig. 2: Semilogarithmic graph showing the time-concentration of difloxacin in plasma of non-treated and probiotic pretreated chickens after the single oral dose of 10mg/kg b.wt.

After oral administration of difloxacin (10mg /kg b.wt.) in chickens with and without probiotic pretreatment, the drug was first detected 0.25 h at a concentration of 0.14±0.01 and 0.11±0.01 µg/ml, respectively (P<0.05). Twenty four hours after oral administration, difloxacin concentrations were 0.10±01 and 0.08±0.0 µg/ml (P<0.05) in plasma of broiler chickens with and without probiotic pretreatment, respectively (Fig. 2). After oral administration, the peak concentration (C_{max}) of difloxacin in plasma was 2.31± 0.17 and 1.8±0.19 µg/ml and it was achieved at maximum time (T_{max}) of 2.5±0.03 h and 2.66±0.06 h (P<0.05) in chickens with and without probiotic pretreatment, respectively. The absorption half-life ($t_{0.5ab}$) was 1.34±0.03 h and 1.43±0.04 h (P<0.05) and the calculated oral bioavailability (F) was 72.82%±5.35% and 64.56%±5.29% in chickens with and without probiotic pretreatment, respectively (Table 2).

Protein binding %: The per cent of *in vitro* protein binding of difloxacin in the serum of probiotic pretreated chicken serum was slightly (9.03) lower than that in normal chicken (10.7%) serum (Table 3).

Tissue residue: The present data revealed that the tissue concentrations of difloxacin at the first-day post last oral dose in muscle, liver, kidney and gizzard, respectively were significantly (P<0.05) lower in probiotic pretreated chickens as compared to normal chickens. This effect persisted up to the 3rd day after cessation of administration.

Table 4: The concentrations (µg/g) of difloxacin in different tissues after last oral dose (10 mg/kg for 5 days) in slaughtered *chickens with and without probiotic pretreatment* (mean±SD, n=5)

Organ	Treatment	Days after slaughter			
		1st	3rd	5th	7th
Muscle	Difloxacin	0.28±0.011	0.06±0.009	ND	ND
	Probiotic+Difloxacin	0.22±0.006*	0.04±0.003*	ND	ND
Liver	Difloxacin	0.46±0.017	0.16±0.011	0.033±0.004	ND
	Probiotic+Difloxacin	0.33±0.005*	0.05±0.002*	ND*	ND
Kidney	Difloxacin	0.71±0.032	0.38±0.018	0.07±0.01	ND
	Probiotic+Difloxacin	0.56±0.016*	0.28±0.004*	0.03±0.001*	ND
Gizzard	Difloxacin	0.25±0.012	0.06±0.009	ND	ND
	Probiotic+Difloxacin	0.19±0.003*	0.03±0.003*	ND	ND

* P≤0.05; compared to difloxacin only treated chickens, ND: not detected.

At the 5th day, difloxacin disappeared from muscle and gizzard of both groups as well as from the liver of probiotic pretreated chickens. Difloxacin concentration was still significantly (P<0.05) lower in the kidney of probiotic pretreated chickens compared to the normal one. Difloxacin disappeared from all of the tested organs by the 7th day (Table 4).

DISCUSSION

Probiotics are live microorganisms that modulate gut microbiota (Wan *et al*., 2016). They confer numerous beneficial effects; including the potential to strengthen the integrity of intestinal epithelium and/or adjust some immune components and modify intestinal barrier function (Wan *et al*., 2016). Moreover, they are known to produce acids and hence lower pH of the environment. They also secret bacteriocin and exert detoxification effects etc. (Pavlova *et al*., 2015). They are now known to exert an important effect on the disposition of many compounds (Stojancevic *et al*., 2014). They are used simultaneously with antibiotics for treatment of some bacterial diseases however; they are not considered an alternative to antibiotics (Pavlova *et al*., 2015).

The 2-compartment open model was the best fitted to explain the kinetics of the drug after administration of the intravenous and oral dose in broiler chickens as indicated by the calculated Akaike's Information Criterion (AIC). Further findings, the 2-compartment open model was used to describe the disposition of difloaxcin in broilers (Abo El-Ela *et al*., 2014) and in Japanese quails (Aboubakr and Elbadawy, 2019), marbofloxacin in chickens (Atef *et al*., 2017), in rabbits (Fernández-Varón *et al*., 2007) and horses (Fernández-Varón *et al*., 2006b).

After IV administration, the half-life of elimination (t½β) of difloxacin was significantly longer in probiotic pre-treated chickens (4.09±0.03h) than non-treated one (3.75±0.02h) and the total body clearance of the drug in probiotic pre-treated chickens (0.44±0.0 mg/(µg/ml)/h) was less than in non-treated chickens (0.48± 0.0 mg/(µg/ml)/h). The higher plasma concentration and the decreased total body clearance of difloxacin may contribute to its prolonged half-life in probiotic pre-treated chickens. Probiotics have been proved to increase in liver protein and plasma protein synthesis through an unidentified signaling mechanism (Harding *et al*., 2008). Moreover, probiotics stimulate intestinal microbiota to synthesize amino acids that are utilized by the host and the bacteria for building of protein (Nath *et al*., 2018). The increased protein synthesis appeared to be not related to plasma protein because plasma protein bounding is slightly lower in plasma of probiotic treated chickens but rather to building protein in muscles being acting as growth enhancers. On the other hand probiotics especially *Lactobacillus* spp. decrease the activity of metabolizing enzymes such as azoreductase, nitroreductase and ß-glucuronidase (Stojancevic *et al*., 2013) that may decrease the degradation of difloxacin and consequently higher plasma concentration, increased AUC and prolonged MRT and prolonged elimination half-life in probiotic-pretreated chickens.

In this work, the half-life of elimination (t½β) of difloxacin in probiotic non-treated chickens (3.75h) was nearly similar to that reported in broilers in other studies (3.70 h, Abo El-Ela *et al*., 2014). However, more prolonged values were reported broilers (6.11 h, Ding *et al*., 2008), in pigs (17.14h, Ding *et al*., 2008) and in goats (5.3h, El-Sayed *et al*., 2013). No previous studies have tested the pharmacokinetics of difloxacin during probiotic pretreatment. The total body clearance of difloxacin in non-treated chickens (Cl; 0.48 mg/(µg/ml)/h) was within the limits reported by previous studies (0.37; l/h/kg Ding *et al*., 2008 and 0.65 l/h/kg; Abo El-Ela *et al*., 2014). However larger amounts were reported in pigs (2.0l/h/kg, Ding *et al*., 2008) and goats (2.04 l/h/kg; El-Sayed *et al*., 2013).

The oral bioavailability of difloxacin in probiotic non treated- chickens was 64.56%. Similar values (68.9%) were reported by Anadón *et al*. (2011). However, higher values (86.2%) were reported by Abo El-Ela *et al*., (2014). In the present work, the oral bioavailability of difloxacin in probiotic pretreated chickens was 72.82% which is significantly higher than that in normal chickens (64.56%). This is probably due to the rapid and complete absorption of difloxacin in probiotic pretreated chickens. No previous studies dealing with the bioavailability of difloxacin during probiotic pretreatment. Probiotics, when administered orally, can support the integrity of intestinal epithelium, enhance intestinal barrier function (Wan *et al*., 2016), increase the length of villi and crypt depth (Sharifi *et al*., 2012) and decrease the pH of the intestines as result of producing organic acids and increase the proteins of the tight junctions (Stojancevic *et al*., 2013). All these effects may positively contribute to good absorption of difloxacin and higher bioavailability and correlate with the reported faster absorption in probiotic treated chickens than non-treated one (t1/2ab; 1.34±0.03 vs 1.43±0.04 h). This is also confirmed by the short tmax (2.5±0.03 h), significantly higher C_{max} (2.31±0.17 µg/ml) and higher value of AUC 0-t (16.32±1.13 µg/ml.h) in probiotic treated chickens than non-treated one (2.66±0.06 h, 1.80±0.19 µg/ml, 13.50±1.24 µg/ml.h, respectively).

Difloxacin attained a C_{max} of 1.8 µg/ml at t_{max} of 2.66 h in probiotic non treated chickens while C_{max} was 2.31 µg/ml reached at t_{max} of 2.5 h in probiotic pre-treated one.

Both the rapid rate of absorption and the higher Cmax contribute to the higher values of AUC_{0-t}, $AUC_{0-\infty}$ and AUMC in probiotic pretreated chickens as compared to non-treated one. The reported C_{max} (1.8 µg/ml) was similar to that recorded in broilers (1 µg/ml, Ding *et al*., 2008; 1.34 µg/ml, Abo El-Ela *et al*., 2014).

The per cent of protein binding in broilers was 10.7%. Lower values were reported in camels (28-43% Abo-El-Sooud and Goudah, 2009) but nearly similar to that reported in goats (13.79±1.02%, Atef *et al*., 2002). The reported in vitro protein bounding of difloxacin in serum from probiotic pretreated chickens was lower than that from non-treated one. This is probably due to decreasing pH by the production of acids by probiotics (Nath *et al*., 2018), since pH is correlated positively with protein binding (Dorn *et al*., 2017).

The residues levels in muscle, liver and kidney were generally lower in probiotic-pretreated chickens indicated that the difloxacin was depleted from tissues faster in probiotic pretreated chickens than non-treated chickens. This is confirmed by the larger rate constant for the transfer of the drug from the tissues to the central compartment (K_{21}; 0.38±0.01 vs 0.36±0.01 h^{-1}) and by the decreased MRT in probiotic-treated chickens. No residues were detected in muscle, liver and gizzard of probiotic pretreated chickens by the 5th-day post-treatment. However, the residues of difloxacin 24 hours after the last oral dose was lower than the recommended MRLs for difloxacin in chicken (300, 1900, and 600 µg/kg in Muscle, Liver and Kidney, respectively (EMEA, 2002) suggesting a withdrawal period of 24 h after the last dose of difloxacin in probiotic pretreated chickens.

Conclusions: Significant alterations of the pharmacokinetic aspects of difloxacin due to concurrent administration of probiotics were reported. Moreover, difloxacin is depleted faster in probiotic pretreated chickens than non-treated one and consequently, the withdrawal period of difloxacin may be re-evaluated when concurrently administered with probiotics.

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Authors contribution: MA: Supervision, Validation, AHA: Supervision, Visualization, Conceptualization and design of the experiments, AD: Resources, supplying reagents and materials, HFM: Investigation, experimentation and analysis of the data, HFM, AHA, MA, writing the manuscript.

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