Original article

EFFECT OF TREATMENT WITH ENROFLOXACIN AND *LACTOBACILLUS* PROBIOTICS ON ABCB1, ABCC2 AND ABCG2 MRNA EXPRESSION IN POULTRY

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Summary

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Poultry feed is often supplemented by *Lactobacillus* probiotics which may alter drug bioavailability by affecting the expression of intestinal ATP-binding cassette (ABC) efflux transporters. Therefore the effect of probiotics, administered alone or in combination with enrofloxacin, on the expression of ABCB1, ABCC2 and ABCG2 mRNAs in chickens was evaluated. Day-old Ross chicks (n=24) were divided in four equal groups. Control group was not treated. The second group received feed with addition of probiotics *Lactobacillus brevis, L. plantarum* and *L. bulgaricus* 5 days after hatching, for 15 days. The third group received probiotics as described above and enrofloxacin at the age of 15 days (10 mg/kg, via drinking water for 5 days). The last group received enrofloxacin at age of 15 days (10 mg/kg, via drinking water for 5 days). Expression of ABC transporters in liver, duodenum and jejunum was determined by qRT-PCR. Down-regulation of ABCG2 mRNA in the liver (P<0.05), its up-regulation in the duodenum (P<0.05) and increased ABCB1 mRNA levels in the jejunum (P<0.05) can be attributed to enrofloxacin treatment. Decrease in ABCC2 mRNA expression in the duodenum can be associated with enrofloxacin administration. The observed changes were related to enrofloxacin administration and to lesser extent to *Lactobacillus* supplementation.

Key words: ABC efflux transporters, broilers, enrofloxacin, *Lactobacillus* probiotics

INTRODUCTION

Oral administration of drugs is the most common practice in the treatment of farm animals reared in groups. Bioavailability of drugs depends on the possibility to pass gastro-intestinal barrier and first pass effect in the liver as initial barriers. There is a lot of evidence that transport proteins are one of the major determinants of the

processes of absorption and distribution of drugs (Ayrton & Morgan, 2001; Van Bambeke *et al*., 2003). ATP-binding cassette (ABC) transporter superfamily includes a number of transmembrane proteins, part of which are P-glycoprotein (encoded by the ABCB1 gene and also referred to multidrug resistance 1), MRP2 (multidrug resistance-associated protein 2, encoded by the ABCC2 gene) and BCRP (breast cancer resistance protein, encoded by the ABCG2 gene). These proteins have been found at high levels in the small intestine and liver of poultry (Haritova *et al*., 2008a; 2010). Fluoroquinolones are examples of P-gp, MRP2 and BCRP substrates and modulators, which has been described as a class-specific behaviour of these drugs (Prime-Chapman *et al*., 2005; Schrickx & Fink-Gremmels, 2008; Guo *et al*., 2013). Enrofloxacin was described as efficiently transporting ABCG2 and P-gp substrate (Pulido *et al*., 2006; Guo *et al*., 2013). The function of P-gp, MRP2 and BCRP was not affected by enrofloxacin in an *ex vivo* model with avian splenocytes (Haritova *et al*., 2007). Danofloxacin showed slight inhibitory effect on the function of P-gp in avian splenocytes and provoked a statistically insignificant increase in all three investigated genes in tissues of healthy turkeys (Haritova *et al*., 2007; 2008b). These changes in ABCB1, ABCC2 and ABCG2 mRNA did not cause alterations in serum levels and pharmacokinetic parameters of orally administered danofloxacin mesylate (Haritova *et al*., 2008b). Pathological conditions such as *E. coli* infection and subsequent treatment with enrofloxacin affected P-gp expression at the transcriptional and translation levels (Guo *et al*., 2014). ABCB1 mRNA and ABCC2 mRNA levels were down-regulated in the gastrointestinal tract of *E. coli* challenged broilers

(Haritova *et al*., 2008a). Treatment of *E. coli* infected poultry with enrofloxacin and danofloxacin resulted in a significant restoration of physiological ABCB1 mRNA levels. Its expression was not completely regained to the levels in healthy chickens particularly in the duodenum and the liver of danofloxacin treated group and in the duodenum and caecum in the group that received enrofloxacin (Haritova *et al*., 2008a). A role of ABC transporters in the excretion of enrofloxacin through gastrointestinal tract, the liver and kidney was suggested in recent investigations with broilers. Higher ABCB1 expression in the intestines of young chickens and in *E. coli* infected broilers resulted in reduced bioavailability of orally administered enrofloxacin (Guo *et al*., 2013; 2014).

Fluoroquinolones interact not only with the ABC transporters in the gastrointestinal tract. Being broad spectrum antibiotics they affect the gut microbiota (Theriot & Young, 2015). Gut microbiota contributes to utilisation of nutrients, affects intestinal morphology, physiology and immunity of poultry (Chae *et al*., 2012; Pan & Yu, 2014). The beneficial role of probiotics on gut microbial community in broilers has been recognised and they are routinely used in poultry. Their administration improves feed intake and digestion and stimulates the immune system (Kabir, 2009). Pathogenic bacteria and probiotic dietary supplementation manipulate these processes in the intestines. Probiotics play an important role in maintaining conditioned microbiota by competitive exclusion and antagonism against pathogens (Wideman *et al*., 2015). As a part of the complex interactions between antibiotics and gut microbiota, probiotics influence drug pharmacokinetics through various mechanisms including

biotransformation, bioactivation, and bio-degradation as well as through up- or down-regulation of the epithelial trans-porters (Stojancevic *et al*., 2014).

Therefore, it is important to investigate the effect of administration of antibiotics and probiotics in order to understand the factors that can have impact on drug disposition. The aim of the present study was to evaluate the administration of enrofloxacin with or without *Lactobacillus plantarum, L. brevis* and *L. bulgaricus* on ABCB1, ABCC2 and ABCG2 mRNA expression levels in Ross 308 broilers.

MATERIALS AND METHODS

Drugs

Enrofloxacin hydrochloride (Baytril 5% injectable solution, KP076SN, 02.2014, Bayer Animal Health GMBH Leverkuzen, Germany) was used for treatment. The drug was administered after dilution of 1 mL drug formulation/1 L water. Medicated water was prepared *ex tempore* in the morning between 7.30 and 8.00 h and in the afternoon between 16 and 17 h.

Probiotic strains

Probiotic strains *Lactobacillus brevis* 51, *L. plantarum* 11 and *L. bulgaricus* 13 (Microbial Collection – Laboratory of Genetics of Probiotic Bacteria, Institute of Microbiology, BAS) were used in the study. The origin of these strains was from traditional dairy products. They were characterised as candidate probiotics according to the *in vitro* criteria of WHO (FAO/WHO, 2002; Danova *et al*., 2012, Tropcheva *et al*., 2013). *Lactobacillus* strains were selected according to their capabilities to survive at the pH values in the gastrointestinal tract of poultry (Tropcheva, 2014). They were cultured in skim

milk (Humana, Holdorf, Germany), lyophilised and stored at –20 °C before administration in the feed. The concentration of the lyophilised products of each strain was as followed: *L. brevis* – 1.6×10^6 CFU.mg^{-1} product, *L. plantarum* 1.06×10^6 CFU.mg^{-1} and *L. bulgaricus* – 0.25×10^3 CFU.mg^{-1}. They had a broad antibacterial activity (Danova *et al*., 2012) and were stable at the concentrations of enrofloxacin that can be attained in the gastro-intestinal tract of poultry (Pavlova *et al*., 2015).

The probiotic strains were administered via feed at a dose of 1 g.kg^{-1} feed from each of lyophilised strain. The feed, supplemented with probiotics, was daily prepared and was administered to the chickens.

Animals and experimental design

The experiments were approved by the Ethical commission for animal experiments at Trakia University, Stara Zagora (Protocol No 65/ 18.10.2013).

One day-old Ross 308 broiler chickens (n=24) were obtained after hatching (Bovans Bulgaria, Chirpan). Birds were given access to feed and water *ad libitum*. They were divided in four groups of six chickens each. The first group remained untreated and served as a control. The second group received feed with probiotics for 15 days. Administration of feed with probiotics started 5 days after hatching. The third group was treated with probiotics as described above in combination with enrofloxacin which was administered via drinking water at a dose rate of 10 mg/kg. The treatment with enrofloxacin started 15 days after hatching and lasted 5 days. The fourth group was treated with enrofloxacin via drinking water at a dose rate of 10 mg/kg for 5 days, 15 days after hatching.

At the day after the end of the treatment with enrofloxacin, 20 days old chickens were euthanised by cervical dislocation and tissue samples of duodenum, jejunum and liver were immediately removed and snap-frozen in liquid nitrogen: clinical examination confirmed the absence of any manifestation of disease at the time of the sample collection. All samples were stored at – 70 °C until analysis.

Real-time PCR analysis

Total RNA was isolated using TRItidy G (Cat. No. A4051, Genaxxon bioscience GmbH, Germany). The quality and quantity of total RNA was determined by ultraviolet absorbance at 260 and 280 nm, and the samples were stored for a short period at – 70° C prior to cDNA synthesis. First-strand cDNA was synthesised using the First Strand cDNA Synthesis Kit (Fermentas Life Science, Cat. No. k1621, Biosystems Ltd., Sofia, Bulgaria) according to the manufacturer's instructions. To a master mixture containing oligo (dT)18 primer and M-MuLV reverse transcrip-

tase, 3 μg total RNA dissolved in sterile nuclease-free water was added. The reaction mixture (total volume 20 μL) was incubated for 60 min at 37 °C, and then the enzyme was heat inactivated at 70 °C for 5 min and the reaction mixture rapidly cooled to 4 °C. Reverse transcription was performed on a Quanta Biotech QB-96 (Quanta Biotech Ltd., Surrey, UK). The cDNA was diluted 1:3 in sterile RNase-free water. Specific primers (Table 1) for the chicken efflux ABC transporters ABCB1, ABCC2 and ABCG2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hexose-6-phosphate dehydrogenase (H6PD) were obtained from Sigma (Sigma-Aldrich, United Kingdom). The primers and the optimal annealing temperatures are presented in Table 1. The real-time PCR analysis was performed with iTaq Universal Sybr Green Supermix (Cat. No. 172-5122, Bio-Rad, Hercules, CA), conducted according to the instructions of the manufacturer. The samples were processed in an Applied Biosystems' StepOnePlus™ Real-Time

Table 1. Chicken specific primers used in the study

Gene	NCBI accession number	Forward primer 5′→ 3′	Reverse primer 5′→ 3′	T_a, °C
ABCB1	NM_204894	GCTGTTGTATTTCCT GCTATGG	ACAAACAAGTGGGCT GCTG	58
ABCC2	XM_421698	CTGCAGCAAAATGAG AGGACAATG	CAGAAGCGCAGAGAA GAAGACCAC	63
ABCG2	XM_004942107 .1	CCTACTTCCTGGCCT TGATGT	TCGGCCTGCTATAGC TTGAAATC	62
H6PD	XM_425746.4	GAGAACCAGCACTTC TTAGAC	GGGTTCAGCAACTCC ACTG	64
GAPDH	NM_204305	GTGTGCCAACCCCCA ATGTCTCT	GCAGCAGCCTTCACT ACCCTCT	65

ABCB1 – gene encoding ATP binding cassette subfamily B member 1, P-glycoprotein; ABCC2 – gene, encoding ATP binding cassette subfamily C member 2, Multidrug resistance-associated protein 2; ABCG2 – gene, encoding ATP binding cassette subfamily G member 2, breast cancer resistance protein; H6PD – hexose-6-phosphate dehydrogenase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; NCBI – the National Centre for Biotechnology Information; Ta – optimal annealing temperature.

PCR System (Applied Biosystems, Thermo Fisher Scientific) and analysed using StepOne™ Software, v. 2.1 (Applied Biosystems, Thermo Fisher Scientific). Following an initial hot-start for 3 min, each reaction went through a PCR cycle with a denaturation step at 95 °C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72 °C for 30 s. After 35 cycles a melting curve was obtained by increasing the temperature with 0.5 °C every 10 s from 65 °C to 95 °C. The analyses were done in triplicate. Gene expression data were presented using the algorithms outlined by Vandesompele *et al.* (2002) and the geNorm manual available on the website https://genorm.cmgg.be/. Expression levels of gene of interests were normalised against reference genes GAPDH and H6PD. Efficiency of each reaction was computed with LinRegPCR 7.0 software.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed with ANOVA followed by Bonferroni's multiple comparison test at significance level P<0.05.

RESULTS

The investigated ABC efflux transporter proteins were found in the studied tissues at mRNA level (Fig. 1, 2 and 3). Treatment of chickens with *Lactobacillus* probiotics with or without enrofloxacin as well as with the antibiotic only led to different changes in mRNA levels of ABCB1, ABCC2 and ABCG2.

ABCB1 mRNA was significantly upregulated in the jejunum (Fig. 2) after enrofloxacin treatment in comparison to groups supplemented with probiotics. In the other tissues its expression was not changed (Fig. 1 and 3).

ABCC2 mRNA was significantly down-regulated in the duodenum in the groups of chickens that received either probiotics or enrofloxacin, or probiotics and enrofloxacin compared to the control group. Similar changes were observed in the liver of chickens treated with enrofloxacin in comparison to the group, supplemented with combination of probiotics and enrofloxacin.

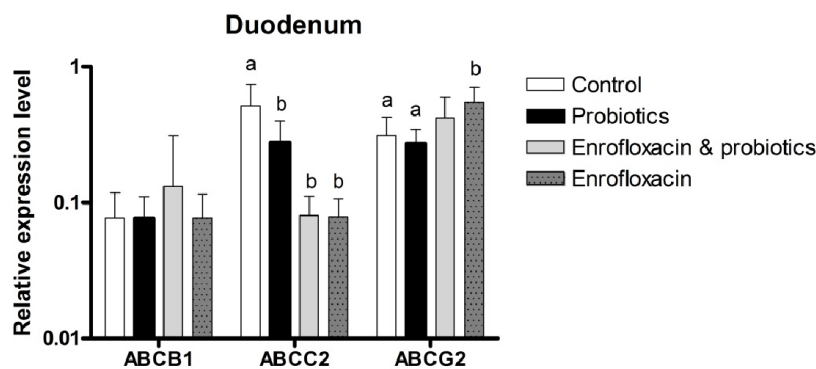ABCG2 mRNA expression was changed in the duodenum and in the liver, and

Fig. 1. Relative expression levels of ABCB1, ABCC2 and ABCG2 mRNA in the duodenum of Ross 308 broilers: untreated (n=6), supplemented with *Lactobacillus* probiotics (n=6), treated with enrofloxacin and *Lactobacillus* probiotics (n=6) and treated with enrofloxacin (n=6). Enrofloxacin was administered orally via drinking water at a dose rate of 10 mg/kg; the letters a and b indicate statistically significant differences between the groups at P<0.05.
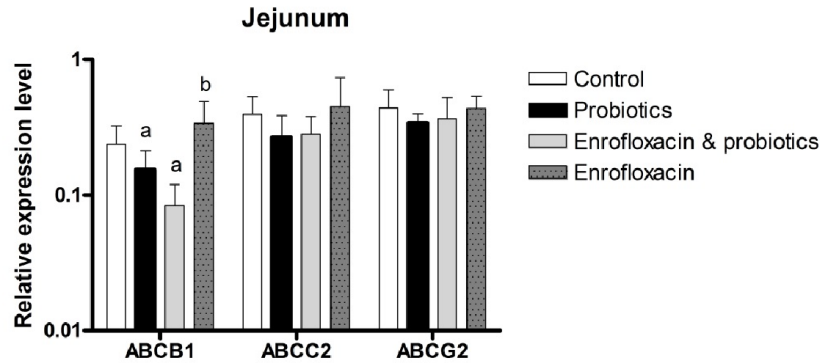
Fig. 2. Relative expression levels of ABCB1, ABCC2 and ABCG2 mRNA in the jejunum of Ross 308 broilers: untreated (n=6), supplemented with *Lactobacillus* probiotics (n=6), treated with enrofloxacin and *Lactobacillus* probiotics (n=6) and treated with enrofloxacin (n=6). Enrofloxacin was administered orally via drinking water at a dose rate of 10 mg/kg; the letters a and b indicate statistically significant differences between the groups at P < 0.05.
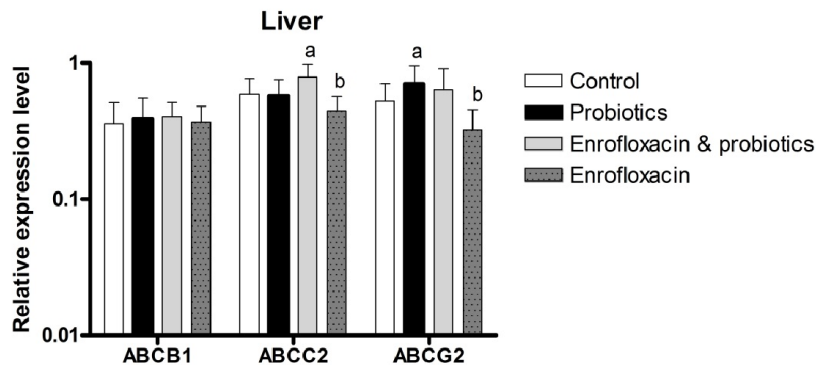

Fig. 3. Relative expression levels of ABCB1, ABCC2 and ABCG2 mRNA in the liver of Ross 308 broilers: untreated (n=6), supplemented with *Lactobacillus* probiotics (n=6), treated with enrofloxacin and *Lactobacillus* probiotics (n=6) and treated with enrofloxacin (n=6). Enrofloxacin was administered orally via drinking water at a dose rate of 10 mg/kg; the letters a and b indicate statistically significant differences between the groups at P<0.05.

was not altered in the jejunum. Enrofloxacin treatment led to significant up-regulation in the duodenum (Fig. 1). In contrary, antibiotic administration provoked down-regulation of ABCG2 mRNA in the liver, which was significant in comparison to chickens, supplemented with *Lactobacillus* probiotics (Fig. 3).

DISCUSSION

Expression and function of ABC efflux transporters can be manipulated by several factors among which are changes in gut microbiota and drug administration. Such changes can provoke variations in drug pharmacokinetics. Enrofloxacin has been described as a substrate for efflux trans-

porter proteins encoded by ABCB1 and ABCG2 (Pulido *et al*., 2006; Guo *et al*., 2013). This drug influences the expression of ABCB1 at protein level after treatment of broilers (Guo *et al*., 2014) but did not modulate the activity of P-gp, MRP2 and BCRP in chicken splenocytes (Haritova *et al*., 2007).

ABCB1 mRNA was found in the duodenum, jejunum and liver of Ross 308 chickens which is in line with previously published data for its abundance in the small intestines and liver (Haritova *et al*., 2010; Guo *et al*., 2013; 2014). The importance of the expression and the function of P-gp for the disposition of enrofloxacin in broilers was demonstrated by Guo *et al.* (2013). They showed that ABCB1 mRNA and its protein P-gp were up-regulated in the jejunum, ileum and liver of one month old chickens if compared to two months old broilers which resulted in reduced bioavailability of enrofloxacin (Guo *et al*., 2013). Additionally, functional studies revealed that pharmacokinetics of enrofloxacin in chickens can be affected by verapamil administration as an inhibitor of this transporter (Guo *et al.,* 2013). ABCB1 mRNA expression levels were not altered in the duodenum and in the liver by any of the treatments but ABCB1 mRNA was significantly up-regulated in the jejunum after enrofloxacin administration if compared to probiotics supplemented broilers. The observed up-regulation of ABCB1 mRNA in the jejunum of Ross 308 broilers was in line with previously published data on the effect of danofloxacin treatment on ABCB1 mRNA levels of turkeys (Haritova *et al*., 2008b). Moreover, enrofloxacin does not exert inhibitory effect on P-gp activity and danofloxacin showed only weak inhibitory effect on the function of this protein (Haritova *et al*., 2007). These data are

supported by the similar disposition of both fluoroquinolone drugs enrofloxacin and danofloxacin in poultry tissues during the course of their continuous oral administration (Haritova *et al*., 2006; Pavlova *et al.,* 2015). Absence of significant changes in ABCB1 mRNA after *Lactobacillus plantarum, L. brevis* and *L. bulgaricus* supplementation with or without enrofloxacin allowed us concluding that adjustment of dosage regimen of the antibacterial drug was not required. These observations agree with a previous study of ours with a similar experimental design which showed that *Lactobacillus* probiotics did not change significantly enrofloxacin pharmacokinetics (Pavlova *et al*., 2015).

Enrofloxacin is a substrate for BCRP and its disposition is mediated also by this efflux transporter, encoded by ABCG2 gene (Merino *et al.,* 2006). ABCG2 mRNA levels in three weeks old Ross broilers in our study were similar to the levels observed in turkeys and four weeks old Ross broilers (Haritova *et al.,* 2010; Su *et al.,* 2014). ABCG2 mRNA was upregulated in the duodenum after enrofloxacin treatment of Ross 308 broilers in the current experiment corresponding to the results obtained after continuous danofloxacin administration in turkeys (Haritova *et al.,* 2010). Taken together these data demonstrate that after oral administration fluoroquinolones are able to increase the levels of ABCG2 mRNA in the duodenum at the concentrations that can be achieved in this tissue. The impact of these changes on enrofloxacin pharmacokinetics has to be evaluated in functional studies with specific inhibitor of BCRP because studies on enrofloxacin pharmacokinetic after continuous drug administration for five days did not reveal any differences in drug disposition (Pavlova *et al.,* 2015). Treatment with enro-

floxacin caused significant down-regula-tion of ABCG2 mRNA in the liver if compared to the group that received pro-biotics but the levels did not differ from these in healthy untreated animals. *Lacto-bacillus* species up-regulate levels of mRNA of this protein in the liver of broil-ers and in mice at different degree (Ange-lakis *et al*., 2012) which is opposite to the effect of pathogens such as *E. coli* and *Eimeria* spp. (Su *et al*., 2014). Adminis-tration of *Lactobacillus* species can have beneficial effect on the maintenance of ABCG2 mRNA expression when they are given alone or in combination with enro-floxacin and thus can be a prerequisite for efficient excretion of its substrates and detoxification of the body.

The last studied efflux transporter was ABCC2 and its mRNA levels observed in the current experiment were close to the previously described expression in healthy chickens and turkeys (Haritova *et al*., 2008b; 2010). Administration of *Lactoba-cillus plantarum*, *L. brevis* and *L. bulgari-cus* and addition of enrofloxacin to probi-otics as well as treatment with the enro-floxacin only, resulted in pronounced sta-tistically significant decrease of ABCC2 mRNA levels in the duodenum. These changes can contribute to hindering the efflux of glucuronides and other conju-gates as well as bile acids in the upper part of small intestines and at the same time can decrease the transport of media-tors promoting inflammation (Tocchetti *et al*., 2016). The effect of probiotics admi-nistered with or without enrofloxacin on the levels of ABCC2 mRNA in the duo-denum of healthy broilers differs from the observed changes in inflamed tissues. Up-regulation of ABCC2 mRNA and its pro-tein in inflammation or cancer of colon was associated with the translocation of eicosanoids and thereby with promoting

of inflammation (Andersen *et al*., 2015). *Lactobacillus plantarum*, *L. brevis* and *L. bulgaricus* mitigate the effect of enroflox-acin on expression of ABCC2 mRNA in the liver because its down-regulation pro-voked by the fluoroquinolone was signifi-cant only in comparison to the group sup-plemented with probiotics plus enroflox-acin. Altogether these data demonstrate that lactobacilli and enrofloxacin are among dietary additives and drugs, respec-tively that are capable to regulate tran-scriptionally intestinal MRP2 expression (Tocchetti *et al*., 2016). Administration of probiotics cannot be associated with chan-ges in ABCC2 mRNA levels which can be observed during inflammation and thus may have positive effect in maintenance of health in the gastro-intestinal tract. However, further experiments with animal models of inflammation are necessary to confirm these effects of lactobacilli probi-otics.

In conclusion, the observed changes in the expression of the studied three ABC efflux transporters were mainly located in the duodenum and the liver, which can be related to enrofloxacin administration and at much lesser extent to *Lactobacillus* supplementation. However, the current study cannot give strong evidence and discussion for the underlying biological mechanisms of the observed changes. It can be used as a basis for further func-tional studies for clarification of the sig-nificance of the observed changes in the studied ABC transporter proteins in efflux of their substrates.

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