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# Therapeutic advantages of the combined use of closantel and moxidectin in lambs parasitized with resistant gastrointestinal nematodes

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ABSTRACT

The serious widespread development of nematode resistance has motivated the use of combined anthelmintic formulations. However, the advantages/disadvantages of the combined use of anthelmintics require further scientific characterization. The goals of the current trial were a) to characterize the pharmacokinetics of closantel (CLO) and moxidectin (MXD) administered both subcutaneously (sc) and orally either separately or co-administered (CLO + MXD) to lambs; b) to compare the nematodicidal activity of both molecules given individually or co-administered to lambs infected with resistant nematodes. Seventy (70) Corriedale lambs naturally infected with multiple resistant gastrointestinal nematodes were involved in the pharmacokinetic and efficacy trials. The animals were allocated into six groups (n = 10) and treated with either CLO, MXD, or with the CLO + MXD combined formulation by both the oral and sc routes. Additionally, an untreated control group (n = 10) was included for the efficacy trial. The efficacy was estimated by the faecal egg count reduction test (FECRT). Higher systemic exposure of both CLO and MXD was observed after the sc compared to the oral administration in lambs. The combined administration of CLO + MXD did not markedly alter their disposition kinetics. At 13 days post-treatment, the administration of both molecules as a single active principle reached efficacy levels ranging between 80% (MXDoral), 84% (CLOoral), 85% (CLOsc), and 92% (MXDsc). The combined oral and sc treatments reached 99% efficacy. No adverse effects were observed after the combined treatment of CLO + MXD, and their co-administration did not show any adverse pharmacokinetic interaction. The combined effect of CLO + MXD successfully restored the maximum efficacy levels, which were not reached by the individual active ingredients.

## 1. Introduction

Sheep production in extensive rearing has been compromised by the pathogenic action of gastrointestinal nematodes. Among gastrointestinal nematodes, *Haemonchus contortus* is by far the most pathogenic nematode parasitizing lambs in Uruguay (Nari et al., 1996; Castells, 2002). For years, control of gastrointestinal nematodes was based on the intensive use of nematodicidal drugs, which resulted in the development of resistance. In most countries where sheep farming holds economic significance, a comparable situation can be observed (Playford et al., 2014; Traversa and von Samson-Himmelstjerna, 2016). In sheep production, *H. contortus* is the main genus involved in anthelmintic resistance to one or more active principles.

Currently, combinations of two or more anthelmintic active ingredients are primarily being used to manage anthelmintic resistance in ruminants, and expand the spectrum of efficacy (Geary et al., 2012). Combination of anthelmintics with a similar spectrum of nematodicidal activity and different mechanisms of action/resistance has been proposed as an alternative parasite control strategy, where a failure of individual drugs is documented (Anderson et al., 1988; Barnes et al., 1995;

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Leathwick et al., 2009; Lanusse et al., 2018). It has been postulated that the ideal situation for the use of nematodicidal combinations is when each of the anthelmintic molecules approaches 100% (Bartram et al., 2012), a situation that has been reported in cattle after the levamisole-ricobendazole treatment (Canton et al., 2018a,b). However, in sheep livestock production systems in Uruguay, is not easy to find sheep herds harboring nematode populations susceptible to the most common anthelmintic drugs. Two exceptions to this general rule could be the anthelmintics closantel (CLO) and moxidectin (MXD). In fact, in the experimental farm of the Secretariado Uruguayo de la Lana (SUL) in which the current study was developed, the *H. contortus* population has been characterized as resistant to albendazole, ivermectin, and levamisole (Suarez et al., 2014), and efficacies above 85% have been observed only for single dose of CLO or MXD (Castells, personal communication).

Drug formulations are available in the veterinary pharmaceutical market combining CLO and a macrocyclic lactone such as ivermectin. The inclusion of CLO in the combination increases the anthelmintic spectrum of ivermectin, since it lacks efficacy to *Fasciola hepatica*. The combined effect against some gastrointestinal nematodes is another advantage of the combined treatment. CLO, a long-acting salicylanilide anthelmintic, mainly targets blood-sucking parasites such as *H. contortus*. In most Uruguay sheep farms, resistance to ivermectin is widespread, but MXD remains more effective against gastrointestinal parasites even in cases of ivermectin resistance (Leathwick et al., 2000; Lloberas et al., 2015; Canton et al., 2018b; 2020; Fazzio et al., 2019; Luque et al., 2021). In this context, the CLO-MXD combined treatment may potentially be a useful strategy, especially when *H. contortus* is the main nematode population involved in gastrointestinal parasitism. CLO and MXD are marketed in oral or injectable forms. While ease of dosing appears to be the primary consideration for selection, how the administration of a combined dose of these compounds impacts absorption, distribution and elimination in sheep remains unstudied. Therefore, there was a need to find dosage combination for administration of CLO and MXD, ensuring maximum efficacy while minimizing potential adverse effects in cases of parasite resistance.

The main goals of the current trial were to characterize the plasma disposition kinetics and the efficacy of CLO and MXD administered either alone or as a combined formulation (both by the oral and the sc route of administration) to lambs naturally parasitized with multiple resistant nematodes (mainly *H. contortus*).

## 2. Materials and methods

### 2.1. Animals

Seventy (70) Corriedale lambs (male and female, 6–7 months old), weighing 24.1 ± 3.5 kg and naturally infected with gastrointestinal nematodes, were involved in this study, which was conducted in the Centro de Investigación y Experimentación "Dr. Alejandro Gallinal" (CIEDAG). This farm, located in Florida, Uruguay, belongs to the Secretariado Uruguayo de la Lana (SUL), and is an experimental unit that has approximately 8000 sheep with a parasite control program based on the intensive use of anthelmintics over the years. Consequently, failure of macrocyclic lactone (ivermectin)(Suarez et al., 2013, 2014), benzimidazoles (albendazole)(Suarez et al., 2011, 2014), and imidazothiazole (levamisole)(Suarez et al., 2014) compounds to control gastrointestinal nematodes has been reported. On day −1, all lambs were individually identified with ear tags and, the number of nematode eggs per gram of faeces (epg) was determined by a modified McMaster technique with a detection limit of 50 epg (Roberts and O'sullivan, 1950). Experimental animals had an average of 5429 epg ranging from 1400 to 12000. Throughout and 40 days before starting the experiment, animals grazed on a natural pasture and had free access to water. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy of the Faculty of Veterinary Medicine, Universidad de la República (UDELAR), Montevideo, Uruguay (protocol number #241–2011).

### 2.2. Experimental design, treatments, and sampling

All parasitized lambs were ranked according to epg counts and then divided into seven groups of 10 animals. On Day 0, each group received the following treatments: Group CLOSC, animals were treated with CLO by the subcutaneous (sc) route at the dose of 5 mg/kg body weight; Group MXDSC, animals were treated with MXD by the sc route at the dose of 0.2 mg/kg body weight; Group CLO + MXDSC, animals were treated with both, CLO and MXD each administered by the sc route (5 and 0.2 mg/kg body weight, respectively); Group CLOORAL, animals were treated with CLO by the oral route at the dose of 10 mg/kg body weight; Group MXDORAL, animals were treated with MXD by the oral route at the dose of 0.2 mg/kg body weight; Group CLO + MXDORAL, animals were treated with both, CLO and MXD each administered by the oral route (10 and 0.2 mg/kg body weight, respectively), and were dosed according to their individual weights. For the efficacy trial, an untreated group was kept as a control. Treatments were performed using commercial formulations of CLO (Saguacid C-L 5%®, CLO 5%, subcutaneous treatment, Laboratorio Dispert, Uruguay and Saguacid C-L 10% ®, CLO 10%, oral treatment, Laboratorio Dispert, Uruguay) or MXD (Cydectin®, MXD 1%, Fort Dodge, Argentina).

The current study involved a pharmacokinetic and parasitological study. For the pharmacokinetic study, seven animals randomly selected from the treated groups were used. Blood samples (5 mL) were collected by venipuncture into 10 mL heparinised Vacutainer® tubes (Becton Dickinson, NJ, USA), before drug administration and at 1.5, 3, 6, 9, 12, 18, 24, 33, 48, 58, 72, 96 h and 6, 8, 10, 13, 17, 22 and 27 days post-treatment. The plasma samples were immediately centrifuged at 3000 g for 15 min and stored at −20 °C until analysis by high performance liquid chromatography (HPLC). For the parasitological study, faecal samples were individually collected from the rectum of each animal (all experimental groups) before treatment (day −1) and at 2, 6, 10, 13, 22, and 28 days post treatment. Epg counts were performed by the McMaster technique modified by Roberts and O'sullivan (1950). Additionally, the nematode genus recovered from parasitized lambs were determined by the identification of the third stage larvae (L3) recovered from pooled faecal cultures (MAFF, 1986) obtained from each experimental group at days −1, 13, 22 and 28 post-treatment.

### 2.3. Estimation of treatment efficacy

The anthelmintic efficacy of the different treatments was assessed by the faecal egg count reduction test (FECRT), calculated according to the formula recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP)(Coles et al., 1992):

$$\text{FECRT } (\%) = 100 \text{ x } (1\text{-}[T2/C2])$$

where T2 is the arithmetic mean epg count in the treated group and C2 is the arithmetic mean epg count in the control group, both at 13 days post-treatment. The 95% confidence intervals were calculated as reported by Coles et al. (1992). In addition, efficacy against different genera was calculated by partitioning the mean faecal egg count of the control group and each treatment group by the proportion of L3 of each genus in the corresponding coproculture (McKenna, 1990). Additionally, since the egg counts observed in the current trial were highly variable, including no egg counts in some animals after treatment, the anthelmintic efficacy and the 95% confidence intervals (CI) were also calculated by the Jeffreys interval (Dobson et al., 2012), where the low confidence interval for a binomial proportion is calculated using the method described by Brown et al. (2001). Here the name 'Jeffreys interval' is used to describe a confidence interval (CI) derived from Bayesian procedures assuming non-informative priors (Dobson et al., 2012). In terms of FECRT the Jeffreys interval define n as the total number of eggs counted

pre-treatment, x the total number of eggs counted post-treatment, p the proportion of resistant eggs (p = x/n) and efficacy (%) = 100 × (1−p).

At 13 and 28 days post-treatment, two (2) animals randomly chosen from groups $CLO_{SC}$, $MXD_{SC}$, CLO + $MXD_{SC}$, $CLO_{ORAL}$, $MXD_{ORAL}$, CLO + $MXD_{ORAL}$, and untreated control were sacrificed by captive bolt gun and rapidly exsanguinated. We selected day 13 according to the range of days suggested by the WAAVP to evaluate the FECRT (Coles et al., 1992; Wood et al., 1995) for macrocyclic lactones. From the parasitological point of view, we wanted to consider at first the efficacy on the populations presents in the animals without considering the elimination of eggs by new infections. In the second instance (day 28) we considered to evaluate the persistence of anthelmintic effect, considering that the prepatent period of gastrointestinal nematodes is approximately 15–23 days (Roeber et al., 2013). Abomasum and different gut sections were identified and isolated and the content analysed to record the number of gastrointestinal nematodes present following the WAAVP guidelines (Wood et al., 1995). Since only two animals per group were sacrificed, the obtained results are only illustrative of parasite burden and remaining parasites after treatment.

Finally, packed cell volume (PCV, hematocrit) was assessed by the microhematocrit technique with blood samples obtained from all animals at day −1, 6, 13, and 28 days post-treatment.

### 2.4. Analytical procedures

Pure analytical standards of MXD, CLO, demethylated CLO (d-CLO), and abamectin (ABM), were obtained from Sigma Chemical Company (Saint Louis, MO, USA).

CLO analysis: CLO was extracted from plasma by a method adapted from Iezzi et al. (2014). Briefly, plasma samples (1 mL) were spiked with d-CLO (as internal standard, IS). After addition of 1 mL of acetonitrile and deionized water (0.25 mL), samples were shaken for 20 min (multi-tube vortexer, VWR Scientific Products, West Chester, PA, USA). The batch of tubes containing the mixtures was placed in an ultrasonic bath (Ultrasound Bath, Lab-Line Instrument, Inc., Melrose Park, OL, US) for 10 min and then centrifuged at 2500 g for 15 min (Jouan®, BR 4i Centrifuge, Saint Herblain, France). The supernatants were recovered and the precipitates obtained from the samples were extracted again with 1 mL of acetonitrile as described above. After that, the supernatants were evaporated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, USA). The dry extracts were reconstituted in 250 μL of mobile phase, and an aliquot of 50 μL was injected into the HPLC system. Experimental and fortified plasma samples were analysed by HPLC to determine the concentration of CLO. CLO and the internal standard were quantified using a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan), fitted with a Kromasil® C18 (5 mm, 250 × 4.60 mm) reverse-phase column (Nouryon, Bohus, Sweden) at 30 °C and a fluorescence detector (Shimadzu; RF 10A XL detector) reading at 335 nm excitation and 510 nm emission. The mobile phase consisted of acetonitrile-water (15:85 v/v) containing 0.05% diethylamine at pH 2.5, adjusted with phosphoric acid, with a flow rate set at 1.5 mL/min. The total run time for the method was 25 min. The analytes were identified with the retention times of 99% pure reference standards. Chromatographic peak areas of each molecule were measured using the integrator software (Class LC 10; Shimadzu Corporation) of the HPLC system. Calibration curves for CLO in plasma were prepared by least-squares linear regression analysis, which showed a correlation coefficient of 0.999. The absolute recovery of CLO from plasma was calculated by comparison of the peak areas from spiked plasma samples with the peak areas resulting from direct injections of standards in the mobile phase. Mean absolute recoveries within the concentration 0.25–160 ng/mL (triplicate determinations) were ≥81.3% in all cases with CV ranging between 6.8 and 7.9%. Precision and accuracy (intra- and interday) were determined by analysis of replicates (n = 5) of blank plasma samples fortified with CLO at 5, 50 and 100 μg/mL. Precision was stated as coefficient of variation (% CV). The interday precision of the method showed CV between 5 and 10%. The limit of detection (LOD) was defined as the mean "noise"/internal standard peak area ratio plus 3 standard deviations (SD). The LOD obtained was 0.1 ng/mL. The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV < 20%, and accuracy of ±20%, and an absolute recovery of ≥70%. The LOQ obtained for CLO in plasma was 0.5 μg/mL. Values below LOQ were not included in the pharmacokinetic analysis.

MXD analysis: MXD was determined by HPLC (Shimadzu chromatography system, Shimadzu Corp., Kyoto, Japan) with spectrofluorometric detection (Detector RF 10, Shimadzu) following the methodology previously described (Lifschitz et al., 1999). Excitation and emission wavelengths were 365 and 475 nm, respectively. A mobile phase composed of water/methanol/acetonitrile (6:40:54, v/v), and a C18 column (Kromasil®, Nouryon, Bohus, Sweden, 5 mm, 250 × 4.60 mm) placed in an oven at 30 °C, were used. A complete validation of the analytical procedures for the extraction and quantification of MXD in plasma was carried out. The compound was identified by the retention time of pure MXD standard, which was 6.3 min. No interference of endogenous compounds was observed after the analysis of blank plasma samples. Method's linearity was tested by constructing analytical calibration curves with blank plasma samples fortified with MXD (range of calibration: 0.1−70 ng/mL). MXD recovery, precision, and accuracy (intra- and interday), LOD and LOQ, was determined as previously described. The analytical calibration curve for MXD in plasma showed a correlation coefficient of 0.996. Mean absolute recovery percentage was 77.9%. The interday precision showed CV between 11.8 and 13.5%. The LOQ was established at 0.1 ng/mL.

### 2.5. Pharmacokinetic analysis of the data

Non-compartmental pharmacokinetic analysis for the plasma concentration versus time curves for CLO and IVM for each individual animal after the different treatments was conducted using the R software version 4.1.2 (R Core Team, 2022). The peak concentration (Cmax) and time to peak concentration (Tmax) were recorded directly from the measured concentration data. The elimination half-life (T½el) was calculated as ln 2/λel, where the terminal elimination rate constant (λel), was calculated by performing regression analysis using data points belonging to the terminal phase concentration-time plot. The area under the plasma concentration-time curve from zero up to the limit of quantification (AUC0-LOQ) was calculated using the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity (AUC0-∞) by dividing the last experimental concentration by the terminal elimination rate constant (λel). The mean residence time (MRT) was calculated as the ratio of AUMC/AUC; where AUMC is the area under the first moment curve.

### 2.6. Statistical analysis of the data

The pharmacokinetic parameters, concentration data, and egg counts are reported as the arithmetic mean ± SD. The pharmacokinetic parameters AUC0-LOQ and Cmax obtained for each drug after administration alone or as a combined treatment were compared by Student's *t*-test. Tmax were compared using nonparametric Wilcoxon two-sample test. The pharmacokinetic parameters Cmax and AUC0-LOQ were used to determine potential drug-drug interactions. The geometric mean ratios (GMR) of the Cmax and AUC0-LOQ for the drug used in a combination/alone and the 90% confidence interval (90%CI) of the GMR were determined. It was concluded that a significant interaction had occurred whenever the 90%CI for a systemic exposure ratio fell entirely outside the equivalence range of 0.8–1.25 (FDA, 2012). Egg counts in each experimental group were compared by ANOVA plus Tuckey test using log-transformed data. Differences in hematocrit values observed among groups and at different days within each group were compared by ANOVA plus Tuckey. In all cases, a value of P < 0.05 was considered statistically significant. The statistical analysis was performed using the

R software, version 4.1.2 (R Core Team, 2022).

## 3. Results

No adverse events were observed in any of the animals from the different experimental groups, showing good tolerability of CLO and MXD used alone or in combination. The plasma concentration profiles for CLO and MXD after their sc administration alone or in combination to parasitized lambs, are shown in Fig. 1a (CLO) and Fig. 1b (MXD). Fig. 2 shows the plasma drug exposure of CLO (a) and MXD (b) administered by the oral route alone or as a combined treatment to parasitized lambs. Regardless of the route of administration, CLO was quantified in plasma up to 28 days post SC and oral treatment. MXD was quantified in plasma up to 28 (sc treatment) or 25 days (oral treatment) post-treatment. Table 1 summarizes the plasma PK parameters for CLO and MXD obtained after the sc and oral administration of each drug either alone or as a combined treatment. The route of administration affected the plasma drug exposure of both CLO and MXD, with significantly lower AUC values observed after the oral treatment (single administration). AUC of MXD given orally was one third of that observed subcutaneously. In the case of CLO the decrease was more pronounced, since this was observed even when a higher dose of CLO (10 mg/kg) was used for the oral treatment. The presence of CLO did not affect the plasma disposition kinetics of MXD after both the SC and oral administration. Similar results were observed for CLO, with the exception of T½el and TMR values which were higher ($P < 0.05$) after the combined sc treatment (Table 1).

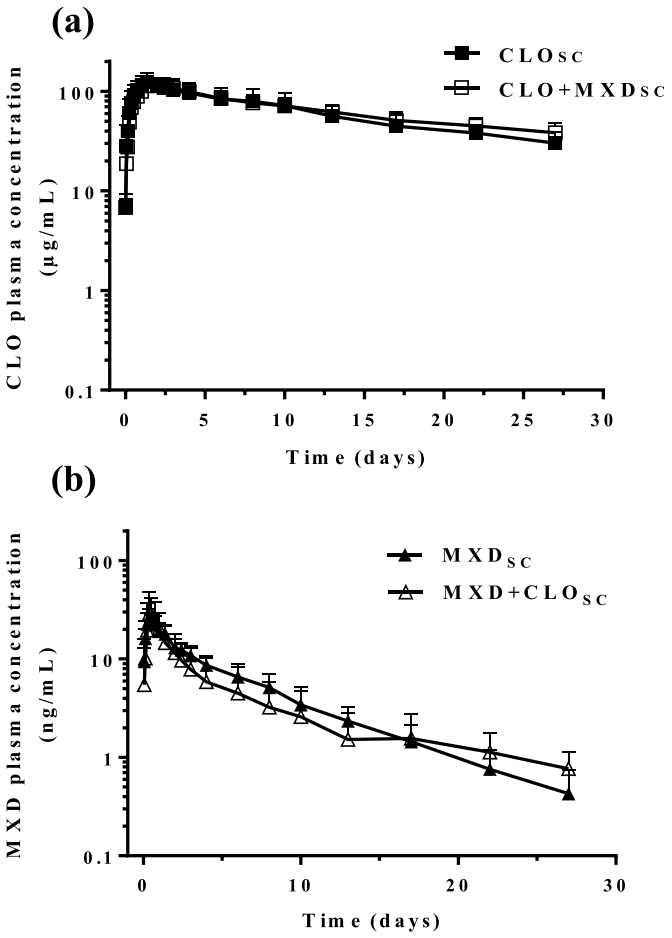


**Fig. 1.** Comparative mean (±SD) (a) closantel (CLO, 5 mg/kg) and (b) moxidectin (MXD, 0.2 mg/kg) plasma concentration profiles obtained after its subcutaneous administration either alone or co-administered to parasitized lambs.

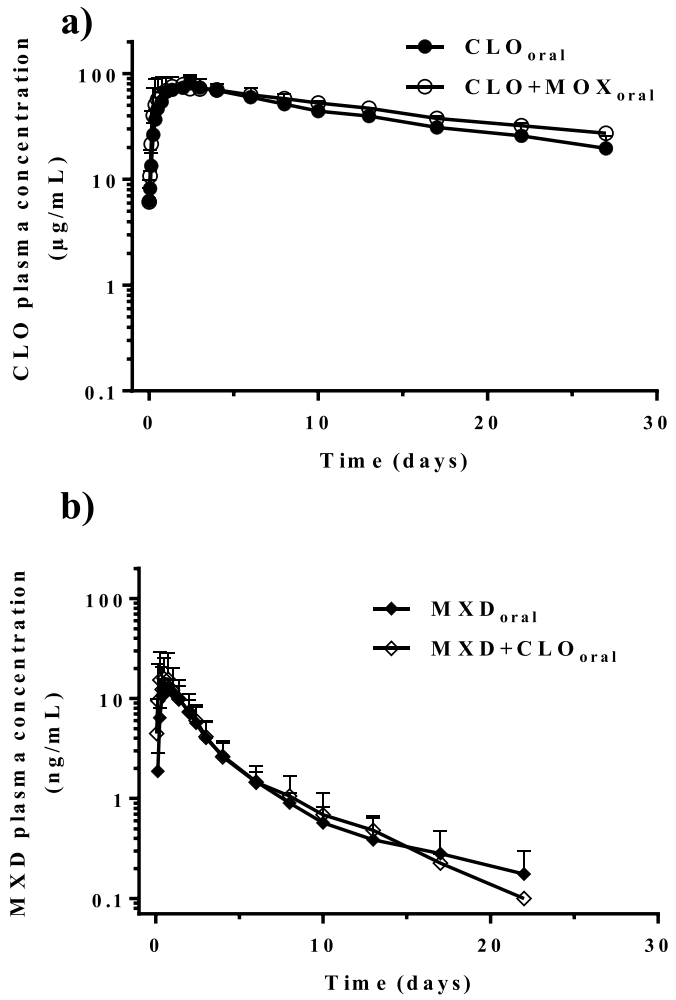


**Fig. 2.** Comparative mean (±SD) (a) closantel (CLO, 10 mg/kg) and (b) moxidectin (MXD, 0.2 mg/kg) plasma concentration profiles obtained after its oral administration either alone or co-administered to parasitized lambs.

The faecal egg counts obtained for all experimental groups before treatment (trial day −1) and at 2, 6, 10, 13, 22, and 28 days post-treatment, including efficacies in treated groups, are shown in Table 2. At day −1, similar ($P > 0.05$) mean epg counts were observed among the different experimental groups (ranging between 5230 and 5630 epg). Faecal egg counts fell early (2 days post-treatment) in all treated groups. The overall efficacy levels observed at 13 days post-treatment (Table 2) indicate the presence of gastrointestinal nematodes resistant to MXD and CLO according to the WAAVP criteria (Coles et al., 1992). This was observed after both the sc and the oral treatments. While MXD alone obtained efficacies between 92% (sc) and 80% (oral), the efficacies of CLO alone ranged from 84 to 85%. The CLO + MXD combined treatment after both the sc and oral administration were the only treatments that reached 100% efficacy at day 13 post-treatment. This high efficacy was maintained up to day 22 post-treatment. In contrast to high initial efficacy of the combined treatment, this substantially decreases at day 28 post-treatment (FECR 50–68%). This decrease was more pronounced when CLO and MXD were administered alone.

The L3 composition (%) observed after faecal culture of pooled samples collected from untreated control animals and the different treated groups at day 0 post-treatment and all sampling occasions (Table 3) showed the predominance of *Haemonchus* spp. (96% in the Control group and between 87 and 98% in treated groups). A minor proportion of other nematode genera (*Trichostrongylus* spp., *Teladorsagia*

**Table 1**
Plasma pharmacokinetic parameters (mean ± SD) for closantel (CLO) and moxidectin (MXD) obtained after its subcutaneous (SC) and oral administration to parasitized lambs either alone or as co-administered (+) treatment.

| Pharmacokinetic parameters | Experimental groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Closantel | | | | Moxidectin | | | |
| | Subcutaneous treatment | | Oral treatment | | Subcutaneous treatment | | Oral treatment | |
| | $CLO_{SC}$ | CLO + $MXD_{SC}$ | $CLO_{oral}$ | CLO + $MXD_{oral}$ | $MXD_{SC}$ | CLO + $MXD_{SC}$ | $MXD_{oral}$ | CLO + $MXD_{oral}$ |
| $C_{max}$ (μg/mL) | 119 ± 24.9$^{a}$ | 114 ± 22.1$^{a}$ | 78.0 ± 14.5$^{b}$ | 78.1 ± 21.3$^{b}$ | 26.2 ± 6.82$^{ab}$ | 33.2 ± 16.9$^{a}$ | 13.1 ± 2.53$^{b}$ | 21.9 ± 15.8$^{ab}$ |
| $T_{max}$ (d) | 1.62 ± 0.42$^{a}$ | 1.91 ± 0.69$^{a}$ | 2.38 ± 0.34$^{a}$ | 1.51 ± 0.69$^{a}$ | 0.47 ± 0.16$^{a}$ | 0.50 ± 0.19$^{a}$ | 0.79 ± 0.09$^{a}$ | 0.54 ± 0.30$^{a}$ |
| $AUC_{0\text{-}LOQ}$ (μg.d/mL) | 1570 ± 419$^{ab}$ | 1645 ± 326$^{a}$ | 1042 ± 170$^{c}$ | 1192 ± 201$^{bc}$ | 118 ± 26.2$^{a}$ | 94.5 ± 54.8$^{ab}$ | 38.7 ± 8.22$^{c}$ | 42.5 ± 17.7$^{bc}$ |
| $AUC_{0\text{-}\infty}$ (μg.d/mL) | 2231 ± 662$^{ab}$ | 2836 ± 822$^{a}$ | 1438 ± 367$^{b}$ | 1888 ± 352$^{b}$ | 123 ± 28.3$^{a}$ | 97.9 ± 57.5$^{ab}$ | 39.9 ± 8.40$^{c}$ | 43.2 ± 17.9$^{bc}$ |
| MRT (d) | 21.9 ± 2.28$^{b}$ | 31.0 ± 6.92$^{a}$ | 20.4 ± 4.62$^{b}$ | 26.9 ± 4.94$^{ab}$ | 5.87 ± 0.76$^{a}$ | 4.98 ± 3.04$^{ab}$ | 3.58 ± 0.90$^{ab}$ | 2.93 ± 1.04$^{b}$ |
| $T_{1/2el}$ (d) | 15.9 ± 2.12$^{b}$ | 22.1 ± 5,38$^{a}$ | 14.4 ± 3.45$^{b}$ | 18.6 ± 3.59$^{ab}$ | 5.6 ± 1.91$^{a}$ | 4.30 ± 2.16$^{ab}$ | 4.00 ± 2.08$^{ab}$ | 2.60 ± 0.84$^{b}$ |

Cmax: peak plasma concentration; Tmax: time to the Cmax; $AUC_{0\text{-}LOQ}$: Area under the plasma concentration vs. time curve from 0 to the limit of quantification; $AUC_{0\text{-}\infty}$: Area under the plasma concentration vs. time curve extrapolated to infinity; MRT: mean residence time (obtained by non-compartmental analysis of the data); T½el: elimination half-life. $^{a, b}$Different letters indicate statistically significant differences ($P < 0.05$) for each drug among different experimental groups.

spp., and *Oesophagostomum* spp.) were present. Based on the L3 composition of faecal cultures, *Haemonchus* spp. was the main genera that survived the combined treatment (Table 3). On day 13 post-treatment, the low number/absence of L3 recovered from faecal cultures makes estimating efficacies by genera impossible. Regarding the adult counts of the main parasite species recovered from lambs (n = 2) of different groups sacrificed at 14 and 28 days post-treatment, the main (number of adult nematodes) nematode species include *H. contortus*, *Trichostrongylus circumcincta* and *Trichostrongylus axei* (abomasum), *T. colubriformis*, and *Cooperia* spp. (small intestine). Furthermore, in the Control group a small number of *Nematodirus* spp. (ranging from 0 to 620), *T. ovis* (ranging from 0 to 12) and *Oesophagostomum* spp. (ranging from 0 to 72) were observed. While *Nematodirus* spp., *Oesphagostomum* spp. and *Trichuris ovis* survive at 13 days post-CLO treatment (both, sc and oral), they were efficiently eliminated by MXD alone or the combined treatment.

The PCV significantly ($P < 0.05$) increased in all treated groups at 13 up to 28 days post-treatment, compared to values observed before treatment (day −1). In the control group, the PCV decreased from a mean of 21.8% observed at day −1 to 16.6% observed at 28 days post-treatment. PCV did not differ significantly between treated groups at any of the sampling points.

Table 4 summarizes the magnitude of exposure ratios obtained from the pharmacokinetic and efficacy trials. The absence of a drug to drug interaction was demonstrated by the GMR combine/single treatments (90%CI) for both CLO and MXD. A significant ($P < 0.05$) reduction in total epg counts was observed after all treatments compared to the control. Furthermore, the efficacy observed after the combined treatment was significantly higher than that observed after the CLO or MXD single treatments.

## 4. Discussion

The current study aimed to explore potential drug-drug interaction between CLO and MXD used as combined treatment in lambs parasitized with multidrug-resistant nematodes. The potential drug to drug interaction studied here includes the pharmacokinetic and pharmacodynamics (drug effect) interactions.

CLO and MXD administered by both the sc and oral routes were characterized by a long plasma drug exposure. In the case of CLO, the prolonged drug exposure is associated with their extensive (>99%) binding to plasma proteins (Mohammed Ali and Bogan, 1987; Hennessy, 1993), which limits their tissue distribution, metabolism, and elimination. On the other hand, the long residence of MXD can partially be explained by their extensive tissue distribution, gastrointestinal recycling (Lanusse et al., 1997), and their low rate of metabolism (Chiu et al., 1990; Zulalian et al., 1994). In most species, MXD is metabolized to only a small degree, and most of the dose is excreted primarily unchanged (90%) in the faeces (Zulalian et al., 1994).

Another common pharmacokinetics feature observed for CLO and MXD after their oral administration to ruminants is their incomplete gastrointestinal absorption. In the case of CLO, this fact justifies the use of a higher dose (10 mg/kg) after the oral administration compared to the sc (5 mg/kg) treatment (Michiels et al., 1987). However, even after the use of a higher oral dose, the plasma drug exposure of CLO (measured as AUC0-LOQ) was 34% lower ($P < 0.05$) than that observed after the sc treatment. Since CLO is not exposed to any significant biodegradation by the ruminal fluid (Hennessy and Ali, 1997), the strong association with the particulate material of the digesta may explain its low enteral bioavailability (Hennessy and Ali, 1997).

As it was previously observed for macrocyclic lactones in sheep (Marriner et al., 1987; Imperiale et al., 2004), goats (Gokbulut et al., 2007), horses (Marriner et al., 1987; Pérez et al., 2003; Saumell et al., 2017) and cattle (Leathwick and Miller, 2013; Canton et al., 2018a,b), the relative plasma availability (AUC0-LOQ) of MXD observed after the oral treatment was significantly lower ($P < 0.05$) than what was observed after parenteral (sc) administration. The AUC0-LOQ after the oral treatment represents only 33% from that observed after the sc treatment. Once again, the high MXD association to the digesta particulate material appears to be a relevant factor limiting its gastrointestinal absorption (Lifschitz et al., 2005). The route of administration (oral or sc) did not modify the time required to peak plasma concentration (Tmax) or the plasma elimination half-life (measured as T½el) of both CLO and MXD (Table 1).

No significant pharmacokinetic changes were observed for CLO and MXD after its co-administration (Table 1). The 90%CI of the GMR for the AUC0-LOQ and Cmax (CLO and MXD) were included in the "no effect" interval (0.8–1.25; FDA, 2006), demonstrating that no pharmacokinetic interaction occurs after CLO + MXD co-administration by both routes. Similarly, Cromie et al. (2006) did not find differences in the plasma pharmacokinetic profile of ivermectin and CLO administered alone or co-administered by the sc route to cattle.

In the current study, the only significant ($P < 0.05$) pharmacokinetic differences were a longer T½el (+39%) and MRT (+42%) observed for CLO after its co-administration with MXD by the sc route. One likely explanation for these pharmacokinetic changes could be based on a drug to drug interaction at the efflux transport level. Mammalian efflux ABC transporters are involved in the efflux of a broad range of xenobiotics and are implicated in the pharmacokinetics of different drugs. P-glycoprotein (P-gp) is a well-studied member of the ABC transporter superfamily, located in the apical side of cells that participate in the ATP-dependent efflux of a broad range of structurally and functionally unrelated compounds out of the cell (Gerlach et al., 1986). P-gp plays a key role in protecting the organism against ingested toxins and contributes to the biliary, urinary and intestinal elimination of different unrelated compounds. It has been demonstrated that CLO interacts with P-gp,

**Table 2**
Nematode egg counts (mean[a] and range) and reduction percentage of faecal egg counts (FECRT[b]) after administration of closantel (CLO) and moxidectin (MXD) by the subcutaneous (SC) or oral route alone or co-administered (+) to parasitized lambs.

| | EXPERIMENTAL GROUPS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $CLO_{SC}$ | | $MXD_{SC}$ | | CLO + $MXD_{SC}$ | | $CLO_{oral}$ | | $MXD_{oral}$ | | CLO + $MXD_{oral}$ | | CONTROL |
| | Mean range | Efficacy (%) [CI] | Mean range | Efficacy (%) [CI] | Mean range | – | Mean range | Efficacy (%) [CI] | Mean range | Efficacy (%) [CI] | Mean range | Efficacy (%) [CI] | Mean range |
| **Day 0** | 5510 | – | 5630 | – | 5440 | - | 5230 | – | 5250 | – | 5590 | – | 5350 |
| | 2600–10200 | | 2200–11200 | | 2600–8200 | | 1600–8100 | | 1400–8400 | | 2000–12000 | | 2300–9300 |
| **Day 2** | 790 [b] | **85** [69–93] | 150 [ab] | **97** [89–99] | 10[a] | **99** | 630 [b] | **88** [77–94] | 240 [ab] | **96** [89–98] | 20[a] | **99** [98–100] | 5420 |
| | 0–2400 | | 0–900 | | 0–100 | [98–100] | 0–1400 | | 0–1000 | | 0–100 | | 900–14800 |
| **Day 6** | 530 [b] | **86** [72–93] | 250[a] | **93** [74–98] | 50[a] | **99** | 550 [b] | **85** [69–93] | 340 [ab] | **91** [72–97] | 40[a] | **99** [95–100] | 3680 |
| | 0–1000 | | 0–1600 | | 0–200 | [96–100] | 0–1700 | | 0–1600 | | 0–200 | | 300–8200 |
| **Day 10** | 460 [bc] | **89** [77–94] | 140 [ab] | **97** [91–99] | 30[a] | **99** | 770[c] | **81** [66–89] | 530 [bc] | **87** [72–94] | 20[a] | **99** [98–100] | 4030 |
| | 0–1100 | | 0–600 | | 0–200 | [97–100] | 0–1700 | | 0–1300 | | 0–100 | | 1200–7700 |
| **Day 13** | 830 [b] | **85** [72–92] | 430 [b] | **92** [85–96] | 10[a] | **99** | 890 [b] | **84** [71–91] | 1140 [b] | **80** [62–89] | 20[a] | **99** [97–100] | 5580 |
| | 0–2200 | | 0–1200 | | 0–200 | [99–100] | 0–1800 | | 100–2600 | | 0–200 | | 2600–11100 |
| **Day 22** | 863 [b] | **82** [67–91] | 975 [b] | **80** [61–90] | 75[a] | **98** | 850 [b] | **83** [73–89] | 888 [b] | **82** [53–93] | 100[a] | **98** [93–99] | 4875 |
| | 200–1900 | | 100–2100 | | 0–300 | [95–100] | 400–1300 | | 0–2500 | | 0–400 | | 1000–9200 |
| **Day 28** | 650 | **84** [57–94] | 1013 | **74** [38–89] | 425 | **89** [66–97] | 1350 | **66** [36–82] | 1525 | **61** [6–84] | 688 | **83** [65–91] | 2450 |
| | 100–2100 | | 0–3000 | | 0–1400 | | 300–2600 | | 200–4900 | | 200–1500 | | 200–6100 |

CI: confidence limit 95%. Nematode egg counts at same day post-treatment with different superscript (a, b, c or d) are statistically different at $P < 0.05$.
[a] Arithmetic mean.
[b] FECRT estimated according to Coles et al. (1992).

**Table 3**
Third stage ($L_3$) larvae composition (%) and reduction percentages of faecal egg counts (FECRT) for *Haemonchus* and other nematode genera after the subcutaneous (SC) or oral administration of closantel (CLO) or moxidectin (MXD), given separately or co-administered (+) to naturally parasitized lambs.

| Genera | Experimental groups | % L3 | Efficacy (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Days post-treatment | | | | |
| | | 0 | 6 | 10 | 13 | 22 | 28 |
| *Haemoncus* spp. | CONTROL | 96 | 81 | 51 | 88 | 60 | 79 |
| | CLO + $MXD_{SC}$ | 94 | * | * | * | * | 51 |
| | CLO + $MXD_{oral}$ | 95 | * | – | * | * | 100 |
| | $CLO_{SC}$ | 98 | – | – | – | 0 | 1 |
| | $CLO_{oral}$ | 88 | * | * | * | 4 | 9 |
| | $MXD_{SC}$ | 94 | * | * | * | 100 | 89 |
| | $MXD_{oral}$ | 87 | * | * | 100 | 100 | 100 |
| Other genera[a] | CONTROL | 4 | 19 | 49 | 12 | 40 | 21 |
| | CLO + $MXD_{SC}$ | 5 | – | – | – | * | 49 |
| | CLO-$MXD_{oral}$ | 5 | * | * | – | – | 0 |
| | $CLO_{SC}$ | 2 | * | * | * | 100 | 99 |
| | $CLO_{oral}$ | 11 | * | * | * | 96 | 91 |
| | $MXD_{SC}$ | 6 | * | * | * | 0 | 11 |
| | $MXD_{oral}$ | 6 | * | * | 0 | 0 | 0 |

- No L3 recovered from faecal cultures.
*Not determined since the low number of L3 recovered.
[a] *Trichostrongylus* spp.; *Teladorsagia* spp. and *Oesophagostomum* spp.

**Table 4**
Changes on the pharmacological exposure and parasitological effects on nematode infected lambs treated with closantel (CLO) and moxidectin (MXD) by the subcutaneous (SC) and oral route, each alone or as a co-administered (+) treatment.

| Experimental groups | Pharmacokinetic study (% change on exposure) | | Efficacy (% change on exposure) | |
|---|---|---|---|---|
| | Pharmacokinetic parameters | *Combined/ alone treatment* GMR[a] (90% CI) | *Treated/ Control* (95% CI)[b] | *Combined/ alone treatment* (90% CI)[3] |
| **$CLO_{SC}$** | $AUC_{0\text{-}LOQ}$ | 0.98↔ (0.75:1.28) | 0.14* (0.07:0.29) | 0.01* (0.00:0.05) |
| | $C_{max}$ | 0.95↔ (0.78:1.16) | | |
| **$CLO_{oral}$** | $AUC_{0\text{-}LOQ}$ | 0.92↔ (0.79:1.08) | 0.15* (0.07:0.31) | 0.02* (0.00:0.07) |
| | $C_{max}$ | 0.98↔ (0.80:1.20) | | |
| **$MXD_{SC}$** | $AUC_{0\text{-}LOQ}$ | 0.73↔ (0.50:1.05) | 0.07* (0.03:0.15) | 0.02* (0.00:0.09) |
| | $C_{max}$ | 1.12↔ (0.71:1.77) | | |
| **MXDoral** | $AUC_{0\text{-}LOQ}$ | 1.03↔ (0.67:1.58) | 0.20* (0.10:0.40) | 0.01* (0.00:0.05) |
| | $C_{max}$ | 1.33↔ (0.78:2.27) | | |
| **CLO + $MXD_{SC}$** | – | – | 0.00* (0.00:0.01) | – |
| **CLO + $MXD_{oral}$** | – | – | 0.00* (0.00:0.01) | – |

[3]Negative binomial distribution means ratio and 90% Confidence interval. When the confidence interval includes a value of 1, the means between treatments are not statistically significant different.
*Statistical significant differences.
$C_{max}$: peak plasma concentration; $AUC_{0\text{-}LOQ}$: area under the concentration vs. time curve from 0 up to the limit of quantification.
[a] GMR= Geometric mean ratio. CI = confidence interval. Symbol: ↔ not determined interaction (the 90% CI surrounding the GMR was within 0.80%–1.25%).
[b] Negative Binomial distribution means ratio and 95% Confidence interval.

increasing the intracellular concentration of rhodamine 123 (a fluorescent P-gp substrate) in P-gp overexpressing cells (Dupuy et al., 2010). Macrocyclic lactones, mainly ivermectin, have been reported as substrate and/or inhibitor of P-gp-mediated transport (Didier and Loor, 1996). The interaction of MXD with mammalian P-gp is much weaker compared with ivermectin (Lespine et al., 2006), which explain, at least in part, the different pharmacokinetic and toxicological profiles observed in mammals for these two related compounds (reviewed by Prichard et al., 2012). However, *in vivo* studies based on co-administration of P-gp inhibitors with MXD indicated that MXD kinetics was somewhat dependent on P-gp or another ABC transporter. For example, Lifschitz et al. (2010) reported that loperamide, a potent P-gp inhibitor, induced changes in the pharmacokinetic behaviour of both ivermectin and MXD in cattle, which may reflect some degree of MXD interaction with efflux transporters. Additionally, the interaction between MXD and breast cancer resistance protein (BCRP), another efflux transporter from the ATP-binding cassette family, was confirmed using cellular transport assays and pharmacokinetic studies in BCRP1 (−/−) and wild type mice (Pérez et al., 2009). In this study, MXD was identified as a BCRP substrate, and its milk BCRP-mediated secretion was demonstrated (Pérez et al., 2009). The pharmacokinetic interaction between CLO and MXD was observed only after their sc co-administration, which could be related to their higher systemic exposure. Independently of the level at which CLO and MXD interact, the magnitude of the interaction is modest and likely could not have any relevant pharmacodynamic (efficacy) consequences.

According to the results obtained after the necropsy of two lambs randomly selected from each experimental group, the overall nematode gastrointestinal burden included *H. contortus*, *T. axei*, and *T. circumcincta* (abomasum), *T. colubriformis*, *Cooperia* spp., and *Nematodirus* spp. (small intestine), and *Oesophagostomum* spp., and *T. ovis* (large intestine). Clearly, *H. contortus* was the most important nematode parasite found in lambs involved in the current experiment at day 0 (87–96% of third stage larvae composition. The nematode population present in the experimental farm (Centro de Investigación y Experimentación "Dr. Alejandro Gallinal", SUL, Uruguay) has been characterized over time as resistant to ivermectin (Castells, 2002; Suarez et al., 2013, 2014), albendazole (Castells, 2005; Suarez et al., 2011, 2014), levamisole (Castells 2005; Suarez et al., 2014) and the ivermectin + albendazole + levamisole combined treatment (Suarez et al., 2014), with mainly *H. contortus* being involved. Similarly, in the current study, *H. contortus* was the main nematode parasite in infected animals and the only nematode population that included individuals resistant to CLO and MXD. However, after both the oral and sc treatment with the CLO + MXD combined treatment, most *H. contortus* were eliminated by the treatment, explaining the high efficacy observed at 13 days post-treatment (100%). Interestingly, a high efficacy of the combined treatment was observed as early as 2 days post-treatment, showing that the treatment rapidly killed nematode parasites or that the egg laying was inhibited shortly after drug exposure. In fact, the temporary suppression of egg output by surviving worms after MXD treatment has been previously described (Sutherland et al., 1999). Efficacy remains high up to 22 days post-treatment only after the combined treatment and decreased after all treatments at day 28 post-treatment. This could be related to the presence of resistant nematodes, since in the absence of resistance, a single dose of CLO (Hall et al., 1981) or MXD (Kerboeuf et al., 1995) protects sheep against susceptible *H. contortus* reinfection for up to 28 and 35 days, respectively.

A higher ivermectin and MXD efficacy against gastrointestinal nematodes of lambs has been observed after their oral/intraruminal treatment (Gopal et al., 2001; Lloberas et al., 2012) compared to that observed after the sc treatment. This greater efficacy has been associated with the higher drug concentration in the abomasal/intestinal content observed after their intraruminal administration (Lloberas et al., 2012, 2013). MXD/ivermectin concentrations measured in *H. contortus* were positively correlated to those observed in the abomasal content (Lloberas et al., 2012, 2013). Additionally, Gopal et al. (2001) reported a higher efficacy of MXD against *T. colubriformis* after the oral compared to the sc administration in sheep. Furthermore, Leathwick and Miller (2013) reported a significantly higher efficacy after oral treatment of MXD (91.1%) than after its sc injection (55.5%) in cattle. However, this does not appear to be the case with MXD in the current work since a similar/higher efficacy was observed after the sc compared to the oral treatment. The oral administration of macrocyclic lactones does not always improve the efficacy against resistant gastrointestinal nematodes. It may depend on the degree of anthelmintic resistance of the involved nematode population (Canton et al., 2018a,b).

The low PCV observed in all experimental lambs before treatment (ranging between 14 and 21.8%) was indicative of the pathogenic effect of *H. contortus* infection. The high efficacy against *H. contortus* observed after treatments determined a sustained increase in PCV over time, which achieved statistical significance at six days post-treatment (Fig. 3) and were close to normality between 10 and 13 days post-treatment. These results indicate that effective control of *H. contortus* would allow rapid recovery from its main pathogenic effect, anemia. Nevertheless, efficacies between 80 and 90% were also enough to allow anemia recovery. This can be explained by the elimination of a significant part of the parasite population, enough to reduce blood loss. Additionally, after CLO treatment, non-blood suckling nematodes "tolerant" to

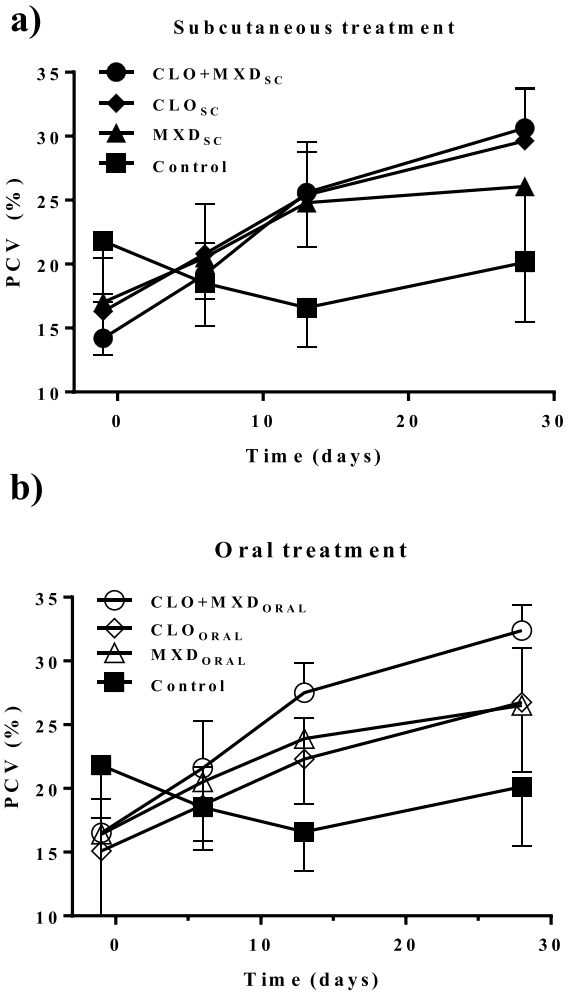


**Fig. 3.** Comparative mean (±SD) packed cell volume (PCV, hematocrit) observed in untreated parasitized lambs (Control) and lambs treated with closantel (CLO) and/or moxidectin (MXD), either alone or co-administered, by the (a) subcutaneous or the (b) oral route.

CLO may contribute to epg and reduced efficacy, without contributing to blood loss.

The findings reported here indicate that the simultaneous administration of CLO + MXD by both the sc and the oral route do not result in any relevant drug to drug pharmacokinetic interaction. The highest efficacy against CLO and MXD resistant nematodes was observed after the combined treatment. However, there is a real risk of populations of multiple-resistant parasites arising after their overuse. In this context, the combined use of CLO and MXD would be only indicated when animals are parasitized with a significant nematode load and in association with refugia-based strategies.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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