

Immune response in Blackbelly lambs to *Haemonchus contortus* and *Trichostrongylus colubriformis* mixed infection in a hot and humid climate

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Abstract. The main objective was to determine the immune response of Blackbelly lambs infected with *Haemonchus contortus*, *Trichostrongylus colubriformis*, or both species. In the first stage, an experimental challenge was performed with 200 infective larvae (L3)/kg body weight (BW) of *H. contortus* (Hct, n = 8). Additionally, eight animals were infected with 500 L3/kg BW of *T. colubriformis* (Tcl), eight lambs with the mixed infection at the same dose (Hct/Tcl), and lambs without infection were the control group. In the second infection, the same lambs were reinfected with high doses: 400 L3/kg BW of Hct and 900 L3/kg BW of Tcl. The third stage consisted of a natural reinfection in grazing. Faecal samples were taken to determine the faecal egg count (FEC) of gastrointestinal nematodes (GINs). IgA was determined in serum and saliva by an indirect Enzyme-Linked Immuno Sorbent Assay (ELISA). The haematological parameters were recorded. With the first challenge, it was not possible to promote an immune response, whereas in the second infection, FEC were higher than 1000 eggs per gram of faeces (EPG) in infected animals. During the third stage, FECs were higher in lambs infected with Tcl. Eosinophils (EOS) did not show differences in the first and second stages, but during grazing the infected lambs had higher counts than the control group ($P < 0.05$). IgA activity values showed the same trend, and lambs infected with Hct/Tcl had a higher response (33% of the positive standard) than groups mono-infected (16.5% and 22.6%, respectively).

INTRODUCTION

Infections with gastrointestinal nematodes (GINs) represent one of the main causes of economic losses in grazing sheep, especially in warm climates, where they proliferate throughout most of the year. The most prevalent nematode species that parasitize small ruminants are *Haemonchus contortus* and *Trichostrongylus colubriformis* (Cruz-Rojo *et al.*, 2012; Macarthur *et al.*, 2013), although an abundance of *Oesophagostomum*

spp., *Strongyloides* spp., *Trichuris* spp., and *Bunostomum* spp. are reported (Zeryehun, 2012).

Studies to know the effect and the impact of GINs have generally been carried out with monospecific infections, mainly using *H. contortus* (González *et al.*, 2008), being a cosmopolitan nematode with a high prevalence in sheep and goats (Domke *et al.*, 2013). This species is considered the most pathogenic as a result of their bloodsucking eating habits (Alba-Hurtado and Muñoz-

Guzmán, 2012). However, in infected animals during grazing, it is very common to find two or more species parasitizing the host (Arece-García *et al.*, 2007; Idris *et al.*, 2012); studies indicating the combined effect of the nematode species are not common.

Selection programs in sheep for increasing resistance against nematodes have also been made towards some particular species, such as resistance studies against *H. contortus* (Alba-Hurtado *et al.*, 2010) or *T. colubriformis* (Kemper *et al.*, 2009). It has been pointed out that when resistance to *H. contortus* is selected, resistance to *T. colubriformis* is also generated (Gruner *et al.*, 2004). Therefore, Blackbelly breed resistance to GINs is indicated (Terefe *et al.*, 2007) and is important to determine the effect of each species on field infections, especially in immunological studies in order to use this information in the selection of animals that resist or tolerate infections towards one or two GIN species.

In the search for biological markers that allow scientists to estimate the effect of each species that parasitize sheep, eosinophilia is an element to be evaluated (Balic *et al.*, 2006). Other options may be the immunological tests that constitute a tool to identify the host immune response against GIN infection (Meeusen *et al.*, 2005). In grazing animals, it is difficult to know the effect of the individual nematode species, because the diagnosis of parasitism is performed by faecal egg count (FEC). For us to know the nematode genus or species that parasitize the animal, coprocultures should be made to obtain the infecting larvae (L3) and estimate the percentage of each species (van Wyk and Mayhew, 2013). However, the results on the number of larvae can be very variable due to factors that affect the development and survival of nematode species. The development of sensitive tests such as immunological assays could help to determine the degree of resistance to the nematode species. According to the results obtained in some studies, it has been suggested that IgA can serve as a marker of susceptibility to GIN infections (Shaw *et al.*, 2012).

The working hypothesis was that sheep infected with the mixture of *H. contortus* and *T. colubriformis* will develop a stronger immune response against both species than lambs infected with a single species. The objective of this study was to determine the cellular and humoral circulating immune response in sheep infected with *H. contortus*, *T. colubriformis*, or with the mixed infection of these species in Blackbelly sheep in warm humid climatic conditions in Mexico.

MATERIAL AND METHODS

Location

The study was carried out in the Regional Center of Chapingo University in Tabasco, Mexico, located at 17° 31' 38" N and 92° 55' 50" W at 60 meter above sea level. According to Kottek *et al.* (2006), the climate in the region is hot and humid (corresponding to rainforest). The annual average temperature is 26.6°C and the precipitation is 3821 mm (CONAGUA, 2017).

Management of animals

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Thirty-two lambs of 12.0 ± 1.5 kg body weight (BW) and three months old were used. The lambs were free of infection (raised in cages with the mother during lactation to assure that there was no infection with GINs). The experiment consisted of three stages.

Stage 1. Experimental infection I. Light challenge

Eight lambs were experimentally infected with a single dose of 200 L3/kg BW of *H. contortus* (Hct). Another eight lambs were infected with a single dose of 500 L3/kg BW of *T. colubriformis* (Tcl). The third group also comprised of eight lambs was infected with the mixture of *H. contortus* and *T. colubriformis* (HctTcl, mixed infection) at the same doses. Finally, a control group of eight lambs born and raised in pens without infection was included.

After infection, lambs were housed for 56 days, then dewormed with levamisole

(Ripercol L-12%, Pfizer, 7.5 mg kg⁻¹ BW) and albendazole (Valbazen 10%, Zoetis, Pfizer, 10 mg kg⁻¹ BW) and left without infection for 15 days to initiate the second stage.

Stage 2. Experimental reinfection II. High challenge

At this stage, the same lambs (14.41 ± 2.01 kg) were reinfected with higher doses. Doses of 400 L3/kg BW of Hct and 900 L3/kg BW of Tci were used. All groups of lambs received the same species reinfection and remained confined in raised pens with the same feeding. This stage lasted 63 days. At the end of the stage, lambs were dewormed with the same anthelmintic and left without infection for 15 days to start the third stage.

Stage 3. Natural reinfection in grazing

In the third stage, all experimentally infected lambs were grazed, and the lambs of the control group were kept in raised cages. Experimentally infected animals were grazed for three weeks in *Brachiaria humidicola* pastures contaminated with GINs to assure a natural infection. Subsequently, the animals were confined for five weeks in cement floor pens.

During the first two stages, the lambs received commercial food with 14% crude protein. The lambs were additionally supplied with dehydrated fodder of *Paspalum conjugatum* and received *ad libitum* water and minerals.

Sampling

Fecal samples were taken directly from the rectum of the animals to determine FECs of GINs, using the McMaster technique with sensitivity of 50 eggs per gram of faeces (EPG) (Cringoli *et al.*, 2004). From each group of lambs during the grazing stage, the larvae were identified from individual coprocultures to corroborate the genus of main GIN in each lamb (Thienpont *et al.*, 1986).

Saliva samples were taken with cotton swabs for the determination of IgA, according to the methodology described by Shaw *et al.*, (2012). Blood samples were also taken in

5 mL Vacutainer tubes (Becton Dickinson, USA). Tubes without ethylene diamine tetra acetate (EDTA) were used to obtain blood serum samples that were collected and stored at -20°C for subsequent immunoglobulin analysis. In the EDTA-blood samples, the percentage of packed cell volume (PCV) was determined with the microhematocrit technique. The concentration of plasma protein (PP) was determined with a refractometer (expressed in g dL⁻¹). The white blood cell count with differential was performed.

Immunoglobulin A

To detect the IgA response in serum and saliva, an indirect Enzyme-Linked Immuno Sorbent Assay (ELISA) with Hct and Tci adult crude antigen (Ag) was performed according to the procedure described by Bowdridge *et al.* (2013) and Andronicos *et al.* (2010), modified by González-Garduño *et al.* (2017). The samples were adjusted to a positive standard to correct the values in each of the plates. In the saliva samples, a larval Ag called hot water larval extract (ELC) was used (Shaw *et al.*, 2012).

Statistical Analysis

The data were analysed with a factorial design considering treatments, stages, and sampling days nested in each of the stages. For this purpose, the statistical package SAS (SAS, 2004) was used. The separation of means was performed using the Tukey procedure.

$$Y_{ijkl} = \mu + \tau_i + \rho_j + \tau^*\rho_{ij} + d(\rho)_{jk} + \tau^*d(\rho)_{ijk} + \delta_k + \varepsilon_{ijkl}$$

Y_{ijkl} = Response variables: EPG, PCV, polymorphonuclear cells, IgA activity. μ = General mean. τ_i = Treatment effect (i = *H. contortus*, *T. colubriformis*, mixture, control). ρ_j = Stage effect (j = First infection, second infection, grazing infection). $\tau^*\rho_{ij}$ = Stage and treatment interaction. $d(\rho)_{jk}$ = Day nested in stage. $\tau^*d(\rho)_{ijk}$ = Day nested in stage with treatment interaction. ε_{ijkl} = Experimental error.

RESULTS

With the light challenge (first stage), the lowest FEC corresponded to the group infected with TcI (52 ± 16 EPG); whereas lambs infected with Hct and HctTcI obtained values ranging 300–380 EPG. Likewise, during the second experimental infection (high challenge), similar FECs ($P > 0.05$) between Hct and HctTcI groups were observed (900 ± 281 EPG and 1481 ± 631 EPG, respectively); both show differences compared to the TcI group (104 ± 47 EPG). During the third stage (when all lambs had natural grazing infections), the highest rates of reinfection occurred in the TcI group (Figure 1).

During grazing (third stage), lambs initially infected with HctTcI presented predominantly with *Cooperia curticei* (53%), while *H. contortus* (36.8–51.1%) was the second most common infective larvae found. In grazing, *C. curticei* was more abundant (23.6–26.7%) than *T. colubriformis* (10.3–19.8%), which became the third most frequent species found (Figure 2).

The PCV was similar in the three infected groups (28.3–29.6%), and these were statistically different ($P < 0.05$) compared to the control group (30.0%). The strongest changes occurred by the sampling date in each stage. In the first stage, the PCV changes were very small in all treatments, whereas during the second stage an increase in the PCV was noticed despite the infection. Conversely, in the third stage, a fall in the PCV occurred due to the natural infection in grazing. The same behaviour was observed in PP (Figure 3).

In circulating eosinophils (EOS), scientists observed differences in favor of infected lambs compared to those without infection ($P < 0.05$), while the three infected groups show similar EOS (Table 1). Leukocytes, neutrophils, and lymphocytes did not show differences between treatments ($P > 0.05$).

The number of EOS was also affected by the study stage ($P < 0.01$). During the first stage, all treatments had similar numbers of circulating EOS. Meanwhile in the second infection, the values increased slightly in the

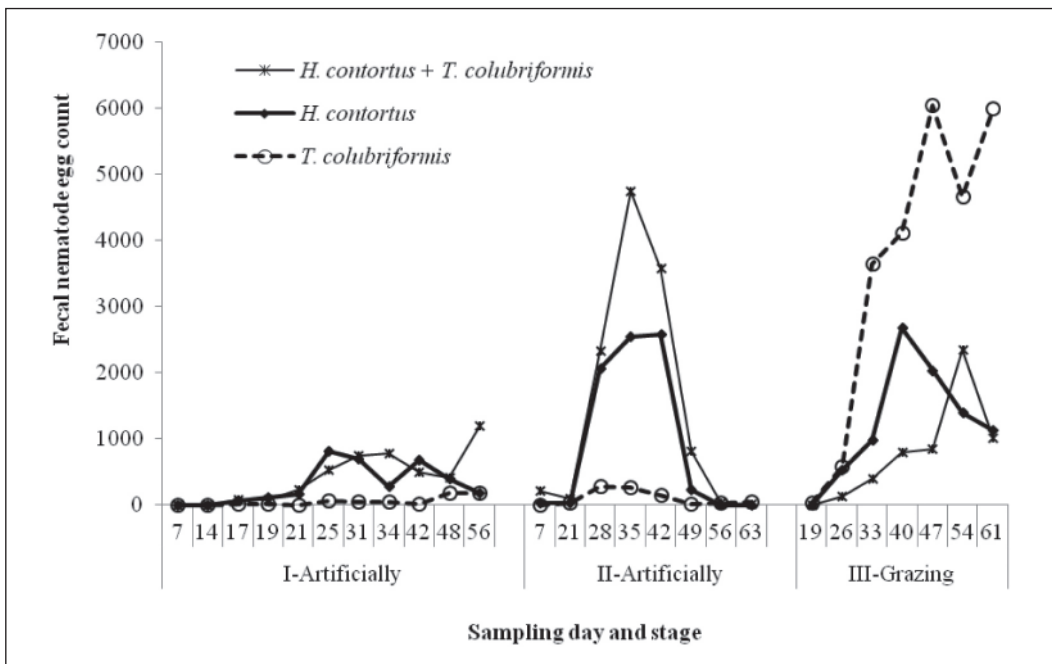


Figure 1. Faecal nematode egg count in Blackbelly lambs experimentally infected with *H. contortus*, *T. colubriformis*, or both species. The third stage was a natural infection in grazing.

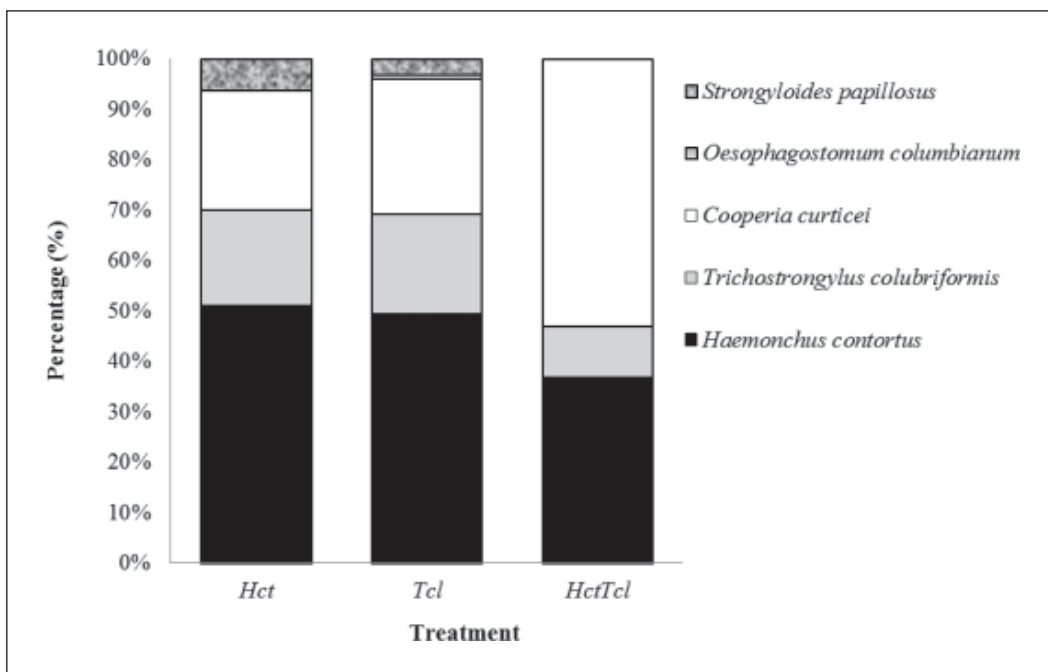


Figure 2. Nematode species found during natural grazing infection (third stage) in Blackbelly sheep. Hct: Preinfected in stage I and II with *H. contortus*, Tcl: Preinfected in stage I and II with *T. colubriformis*.

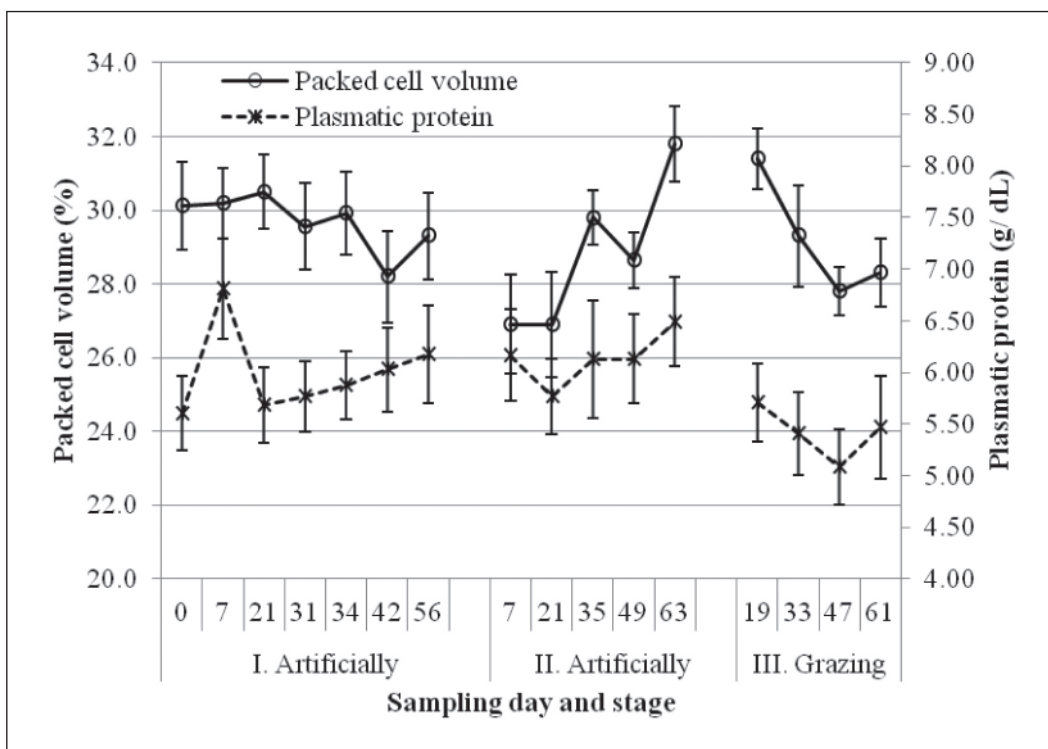


Figure 3. Effect of sampling day and infection type (stage) on packed cell volume (PCV) and plasma protein (PP) of lambs infected with *H. contortus*, *T. colubriformis*, or both.

Table 1. Differential count of peripheral leucocytes of lambs infected with *H. contortus*, *T. colubriformis*, and the combination of species

Cellular group	Mixed infection			Control Mean \pm SD
	<i>H. contortus</i> Mean \pm SD	<i>T. colubriformis</i> Mean \pm SD	(<i>HctTcl</i>) Mean \pm SD	
Leucocytes (x 10 ³ / μ l)	11.6 ^a \pm 3.4	10.7 ^a \pm 3.3	11.1 ^a \pm 3.8	10.9 ^a \pm 3.7
Neutrophils (x 10 ³ / μ l)	4.6 ^a \pm 2.8	4.3 ^a \pm 2.0	4.6 ^a \pm 2.3	4.2 ^a \pm 2.5
Eosinophils (x 10 ³ / μ l)	0.37 ^{ab} \pm 0.27	0.58 ^a \pm 0.38	0.43 ^a \pm 0.36	0.18 ^b \pm 0.15
Lymphocytes (x 10 ³ / μ l)	6.6 ^a \pm 2.5	6.1 ^a \pm 2.7	5.8 ^a \pm 2.4	6.4 ^a \pm 2.5

Different letters in each row indicate differences (P < 0.01).

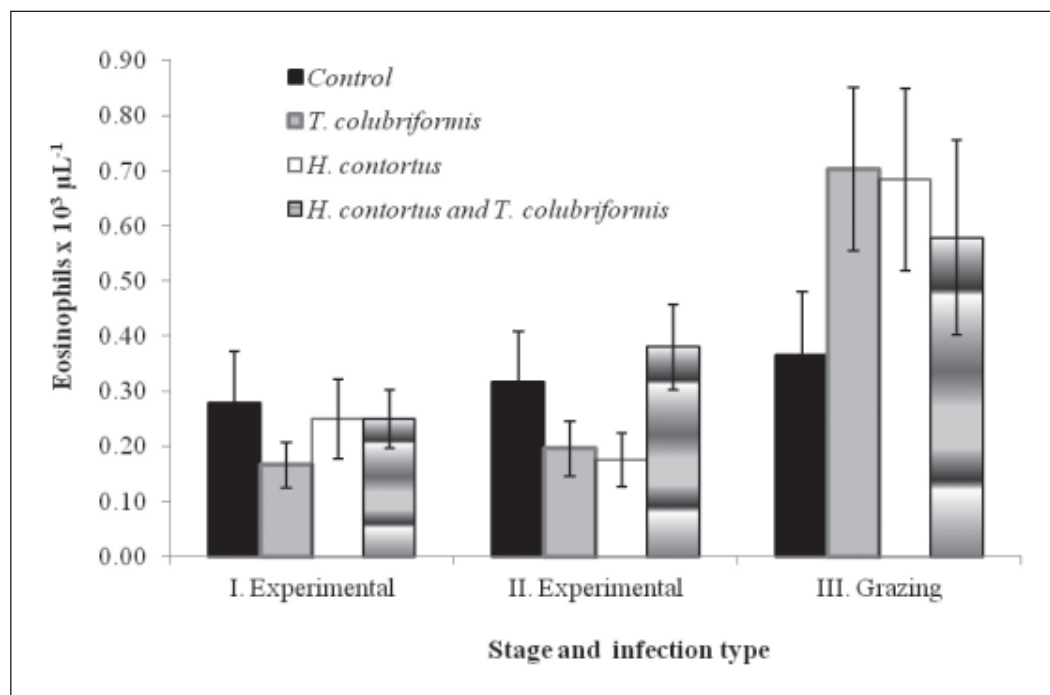


Figure 4. Peripheral eosinophils count in Blackbelly lambs infected with *H. contortus*, *T. colubriformis*, or both during three stages of study.

mixed infection group without showing differences with the other groups, whereas in the third stage, a considerable increase was observed in the infected lambs that were different from the control group (Figure 4).

Neutrophils and lymphocytes were affected by the sampling stage and sampling date. In the first stage, the neutrophils decreased after the seventh day of infection and were maintained at the same level during this stage. In the second infection, they increased gradually from the beginning of

the stage, whereas during the grazing infection (stage III), neutrophils presented oscillations between the samples. Lymphocytes increased slightly at the end of the first infection, in the second infection the number decreased, and during the third infection they remained constant (Figure 5).

During the first and second experimental infections, IgA activity using crude *H. contortus* Ag, did not show response in all treatments. IgA levels did not exceed 5% with respect to the positive standard used.

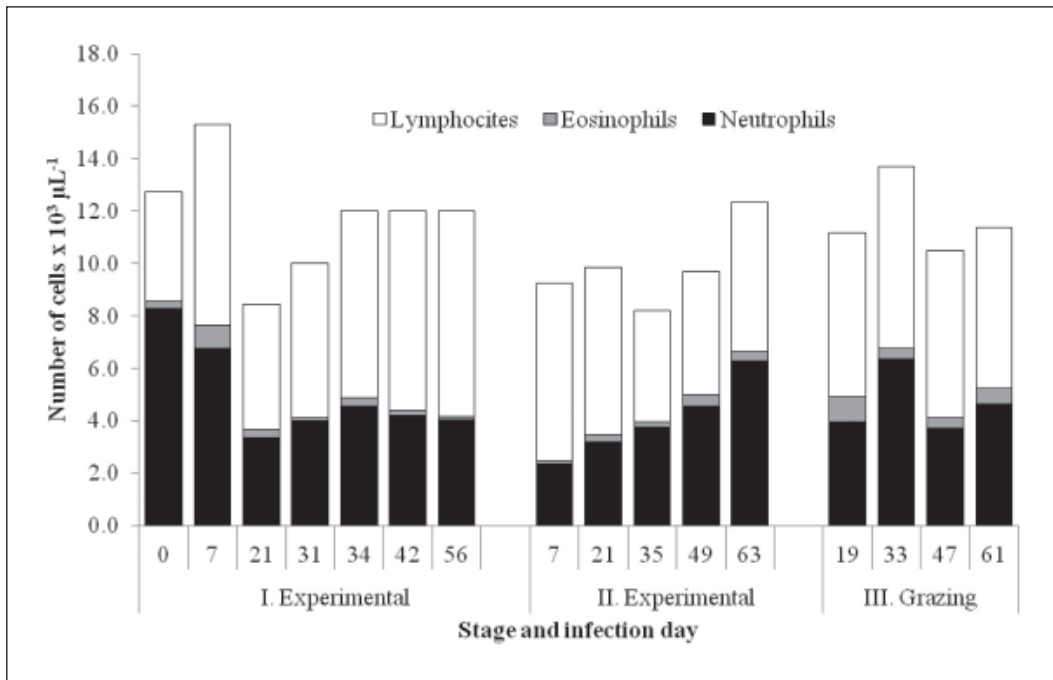


Figure 5. Differential count of peripheral leukocytes in Blackbelly sheep infected with *H. contortus*, *T. colubriformis*, or both during three stages of study.

However, during grazing (third stage), all lambs had increased IgA activity. The Hct/Tcl group had the highest response (33% of the positive standard), whereas the Hct and Tcl groups showed similar IgA activity (16.5% and 22.6%, respectively) (Figure 6a). Using the Ag of *T. colubriformis*, similar results were obtained but with lower IgA activity (Figure 6b).

During grazing, all animals showed an increase in IgA activity with differences compared to control animals. By this reason, IgA activity in both serum and saliva showed a difference between treatments ($P < 0.01$). The IgA activity of lambs with mixed infection was higher than those with monoinfection, and the mean values of infected animals were higher than the control group (Table 2).

DISCUSSION

In the first stage, FECs were very low, because the lambs were infected only with light challenge and this infection did not

promote a strong immune response, as observed in the immunological variables studied. The low number of peripheral EOS and the low levels of IgA are associated with the high FECs in the second stage, specifically in Hct and Hct/Tcl groups, which show greater than 1000 EPG. In the present study, it was shown that small challenges did not cause the acquisition of resistance, while with higher challenge; lower counts were obtained at reinfection for the development of acquired resistance (González-Garduño *et al.*, 2014). The results obtained in our study were probably due to the fact that only single infections were performed in the first and second stages, which could have a similar effect on the truncated infections (only four days) that would not allow immune development in lambs (Emery *et al.*, 1992) and probably the threshold of immune development was not reached.

FECs were associated with the nematode species that infected the animal, but not to the immune levels in both EOS counts and IgA levels during the first and second stages. The highest contribution to FEC in the three

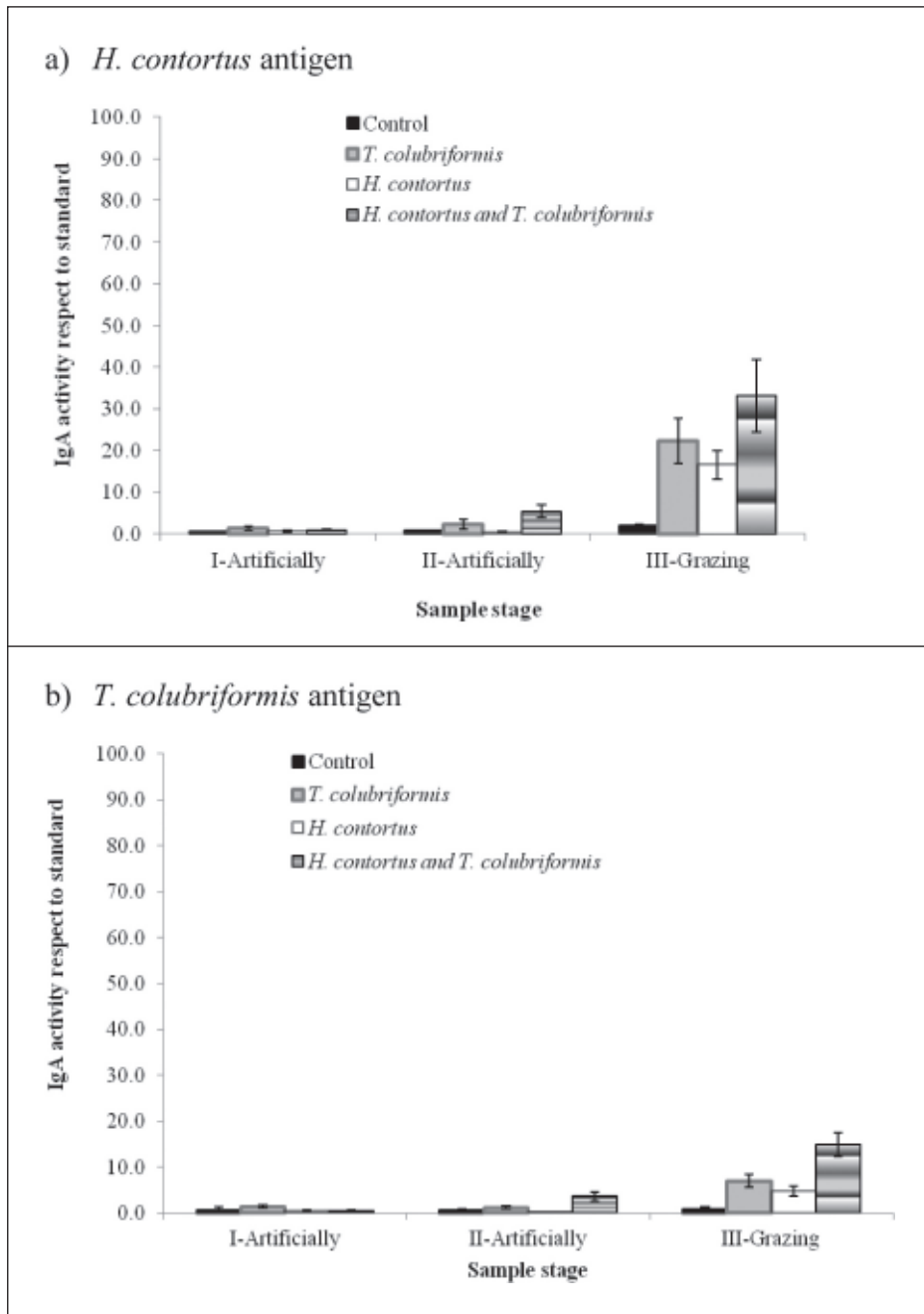


Figure 6. IgA activity against crude *H. contortus* and *T. colubriformis* antigens in Blackbelly sheep in during three stages of study.

stages was due to the infection with Hct. The challenges of 12,000 L3/kg BW with this species in the Manchega sheep breed led to FECs of 1700 EPG at 35 and 40 days and a maximum of 3600 EPG at 60 days (Angulo-Cubillán *et al.*, 2010). On the other hand, the

contribution of Tc1 to FECs was small, as was observed in the maximum expulsion in the first and second stages (183–283 EPG). Also, it has been indicated in values in Merino sheep infected with 10,000 L3/kg BW of Tc1 + 10,000 L3/kg BW of *Teladorsagia*

Table 2. IgA activity against adult crude antigen in Blackbelly sheep infected with different species of gastrointestinal nematodes

Antigen	<i>HctTcl</i>		<i>H. contortus</i> (<i>Hct</i>)		<i>T. colubriformis</i> (<i>Tcl</i>)		Control	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Serum								
<i>H. contortus</i>	10.62 ^a	3.0	3.76 ^{bc}	1.1	6.99 ^{ab}	1.9	0.82 ^c	0.1
<i>T. colubriformis</i>	5.20 ^a	1.3	1.31 ^{bc}	0.4	2.76 ^b	0.7	0.61 ^c	0.1
Saliva								
<i>H. contortus</i>	13.30 ^a	2.3	7.23 ^{bc}	0.9	10.39 ^{ab}	1.2	5.96 ^b	1.2
<i>T. colubriformis</i>	3.91 ^a	0.6	2.49 ^{ab}	0.4	3.16 ^{ab}	0.4	2.06 ^b	0.4

Different letters in each row indicate differences ($P < 0.01$).

circumcincta, with results of 57–302 EPG in resistant animals and 439–1107 EPG in susceptible animals (Kemper *et al.*, 2010). Gruner *et al.* (2003) have indicated a similar response in Blackbelly sheep infected with 20000 L3/kg BW of *Tcl*.

In response to the low single-dose challenges in the first and second experimental infections, PCV and PP were not affected. In addition to the low rate of infection, the feed provided sufficient nutrients to maintain normal haematological values, similar results to those indicated in lambs (Acharya *et al.*, 2015). The reduction of PCV and PP during grazing seems to be an effect of the higher incidence of *H. contortus* in pastures, as was observed in other study (Angulo-Cubillán *et al.*, 2010). The reduction in PCV and PP has been associated with high FECs especially in lambs infected with *H. contortus* (Amarante *et al.*, 2004). Both PCV and PP have also been used to characterize resistant animals with higher values than susceptible lambs (Zaros *et al.*, 2014).

In the grazing infection, the main nematode found was *H. contortus*, indicating (that even in the *Hct* and *HctTcl* groups) immunity was not sufficient to avoid infection with this species. However, in grazing, both groups had lower FECs than the preinfected *Tcl* group. These results suggest that the doses used in this study of *T. colubriformis* did not lead to reductions in the FECs during grazing, which was explained by the great abundance of *H. contortus*.

In the present study, the small GIN infection did not promote an increase in the EOS count in a peripheral way; and, by this reason, the number of EOS was similar in the two first stages, whereas during the grazing infection (third stage) an increase in peripheral EOS counts occurred, which can be attributed to continuous infection. In our case, there were no differences between the infected groups, but differences with the control animals occurred ($P < 0.05$). EOS counts have been one of the main study variables in the search for immunological resistance to parasites, because they are the first defence line in the mucosal tissues and react locally after 7 days (Bowdridge *et al.*, 2015). EOS counts could be detected in blood, because they increase peripherally at 14 days post-infection (Santos *et al.*, 2014). Another reason for the increased number of EOS in the third stage could be the age of lambs, because age is one of the most important factors in the maturation of the immune system. There are previous studies that indicate changes as the time elapses after a challenge with GINs (Andronicos *et al.*, 2014). Sheep aged 16 or 28 months are no more able to resist short-term primary nematode challenges than lambs, but respond more rapidly and suppress nematode egg counts in their faeces.

The neutrophil counts showed differences in the stages – in the second stage with a high challenge the neutrophils increased with time. This agrees with another study in which differences in white

cell counts, such as neutrophils and lymphocytes, have been observed after infection with GINs (Andronicos *et al.*, 2014). Neutrophils have been associated with natural resistance to GINs. Higher values in circulating neutrophils have been indicated in hair sheep (3018 cells μl^{-1}) compared to wool sheep (1818 cells μl^{-1} ; Bowdridge *et al.*, 2015).

In this study, EOS counts were highest in the third stage, showing greater importance in the immunological response against the parasites (Mantovani *et al.*, 2011), because activity against GINs occurred (Reinhardt *et al.*, 2011). In addition, together with other response cytokines (e.g., IL-13, IL-4, and IL-5), they stimulate the expulsion of GINs from the site of infection (Karrow *et al.*, 2014).

The IgA activity values during the two first infections were similar to those of the non-infected animals. The group of lambs infected with both species reached only 5.4% of the standard, which was slightly above the cut off value (5.09%). The low IgA response at the peripheral level during the first two infections was probably because this immunoglobulin is produced locally on the surface of the gastrointestinal mucosa (McRae *et al.*, 2015); and, therefore, it was not possible to detect in serum. With a simple infection, the FECs increased after 25 days postinfection (dpi) and were reduced after 35–42 dpi. At 58 dpi, the FEC was less than 400 EPG (possibly IgA was acting locally along with other cellular mechanisms that allowed the reduction of FEC). While in the third phase, after two previous exposures, the IgA level increased significantly and the FEC was highest, as a result of the continuous infection during grazing.

Another study with Santa Ines lambs reported one increase in IgA levels in serum at four weeks postinfection; however, the differences with respect to uninfected animals started at six weeks (Cardia *et al.*, 2011). The differences regarding the present study are probably because Santa Ines lambs were infected twice a week with 2500 L3/kg BW of Tc1, whereas in our study there was only one single infection. However, during the third stage in which the animals were infected

grazing, the IgA activity was comparable to that found in the Santa Ines lambs, reaching values close to 60% respect to the positive standard.

In the third stage during grazing at 19 days postinfection, IgA levels increased markedly and correspond to a reinfection response as observed in a study by Martínez-Valladares *et al.* (2007), which indicated that IgA levels increased rapidly in sheep reinfected with *T. circumcincta*. Halliday *et al.* (2007) also reported similar response values in IgA production against the same nematode species.

At 21 days postinfection, one study found that the IgA values against *H. contortus* Ags were increased during the first infection and remained high up to 35 days, at which time they were dewormed. The animals reinfected with *H. contortus* or *H. placei* increased their IgA values seven days later, but the animals in which it was their first infection presented IgA values with respect to a smaller standard (Santos *et al.*, 2014).

CONCLUSION

The contribution of *T. colubriformis* to the number of nematode eggs was very small compared to that of *H. contortus*. The low challenge in the first infection did not stimulate the immune response, and during the second infection, there was no response in the IgA nor in the production of eosinophils. However, grazing with the constant challenge developed the immune response acquired by increasing the levels of IgA and eosinophils, especially in the lambs preinfected with the mixture of *H. contortus* and *T. colubriformis* (mixed infection).

Declaration of interests

The authors declare that they have no conflict of interest.

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