Immune response and performance of growing Santa Ines lambs to artificial *Trichostrongylus colubriformis* infections

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ABSTRACT

This study was carried out to evaluate the immune response and the impact of *Trichostrongylus colubriformis* infections on the performance of growing Santa Ines lambs. Thirty male lambs, 3–4 months of age, were maintained in individual pens and restrictively randomised by weight into 3 treatment groups: (1) infected group, artificially infected with 2500 *T. colubriformis* larvae, three times a week, for 13 weeks, and fed *ad libitum*; (2) Pair-Fed Group, non-infected and fed with the same amount of food consumed by the infected animal of the same class on the previous day; and (3) control group, non-infected and fed *ad libitum*. Refused feed was weighed daily to assess the food intake of each lamb. Animals were weighed weekly and blood and fecal samples were collected. At the end of the trial, all lambs were euthanized to determine worm burden and collect intestinal tissues and mucus samples for histological and immunological analysis. The infected group presented eosinophilia, increased number of inflammatory cells in the mucosa, in addition to an increased production of specific immunoglobulins against *T. colubriformis*, which partially prevented the establishment of infective larvae. As a consequence of parasitism, the infected lambs presented reduced serum albumin concentrations and demonstrated severe small intestine lesions, such as villous atrophy and epithelial erosion, which impaired the digestion and absorption of nutrients, causing a significant loss in performance. The infected group presented a 37% reduction in daily weight gain (107.26 ± 10.8 g/day), when compared with the control group (171.07 ± 7.15 g/day). The infected lambs also demonstrated the worst food conversion, i.e., each animal needed to consume on average 10.05 ± 0.52 kg of food to gain 1 kg live weight. The voluntary hay intake depression in infected animals was small, and statistical difference ($P < 0.01$) was seen only on two occasions (ninth and 12th weeks), in comparison with the control group. In conclusion, Santa Ines lambs infected with *T. colubriformis* demonstrated a reduction in performance, caused by the damages produced by the adult nematodes in intestinal mucosa, and also by the immunopathological changes associated with the infection.

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1. Introduction

Infections caused by gastrointestinal nematodes constitute the most important animal health issue for the sheep industry in Brazil, due to reduced productivity, mortality of animals and great expenses with veterinary products and labor. This situation has worsened with the indiscriminate use of anthelmintics as the exclusive method used to control gastrointestinal nematode infections, consequently leading to the selection of resistant nematode populations (Thomaz-Soccol et al., 2004). *Haemonchus contortus* and *Trichostrongylus colubriformis* are the most important

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gastrointestinal nematodes of sheep in Brazil (Rocha et al., 2008).

As *H. contortus* is a highly pathogenic parasite, it has been the focus of most of the studies carried out with sheep breeds raised in tropical and sub-tropical areas. In contrast, despite the usually high intensity of infections, little attention has been paid to *T. colubriformis*. Infections by this nematode constitute an important cause of economic loss for farmers in breeding small ruminants in the several regions of the world (O'Connor et al., 2006). In heavy infections, *T. colubriformis* can cause severe enteritis, characterized by extensive villous atrophy, crypt hypertrophy, intestinal epithelium erosion, infiltration of leukocytes and great serum protein exudation for intestinal lumen (Taylor et al., 2010). As a consequence of these infections, there is impairment in the digestion and absorption of nutrients (Cantacessi et al., 2010). Studies on wool sheep have shown that infected animals may present diarrhea, reduction in food intake, decreases in live weight gain and wool quality (Roseby, 1973; Horton, 1977; Steel et al., 1980; Symons, 1983; Kimambo et al., 1988; Kyriazakis et al., 1996).

The Santa Ines hair sheep is the predominant breed of sheep encountered in most of the Brazilian territory (Santos, 2007). The major reason for the success of this breed, is its capacity to resist *H. contortus* infection, in comparison with sheep breeds of European origin also raised in Brazil (Rocha et al., 2005; Bricarello et al., 2005; Costa et al., 2007; Amarante et al., 2009).

However, Amarante et al. (2004) compared Santa Ines, Ile de France and Suffolk lambs that were naturally infected by gastrointestinal nematodes and observed a higher resistance to *H. contortus* and *Oesophagostomum columbianum* infections, but all three breeds had similar *T. colubriformis* worm burdens. Similar results were obtained in the comparison between Florida Native (resistant) with Rambouillet (susceptible) lambs (Amarante et al., 1999), as well as in the comparison of Gulf Coast Native (resistant) with Suffolk (susceptible) lambs (Bahirathan et al., 1996). These results indicate that, in those "resistant breeds", there are differences in the host–parasite interaction regarding the different nematode species, i.e., the immune response possibly acts with higher efficiency against *Haemonchus* spp. infection than against *Trichostrongylus* spp.

The losses generated by infection with *T. colubriformis* reinforce the importance of research involving this neglected nematode in South America, coupled with the lack of studies on this parasite in hair sheep was the motivation for the present experiment, which was carried out to evaluate the immune response and the impact of *T. colubriformis* artificial infections on the performance of Santa Ines growing lambs.

2. Materials and methods

2.1. Animals

Thirty lambs were raised indoors with their mothers since birth and were weaned at 60 days of age. After that they were transferred to individual pens with a concrete floor that was washed with water under pressure every two days.

During an initial two week-period of adaptation, the animals were vaccinated against clostridioses (Sintoxan Polivalente®–Merial S/A, Brazil) and fecal examinations were carried out that showed that 15 lambs were shedding trichostrongylid eggs with a maximum of 200 eggs per gram (EPG). One of the animals also passed 200 EPG of *Strongyloides* spp. and *Eimeria* spp. oocysts were also sporadically observed in some animals, however, in small quantities. Larvae from *Haemonchus* spp. (78%), *Trichostrongylus* spp. (12%) and *Cooperia* spp. (10%) were found in fecal cultures from these lambs. For this reason, all lambs were orally treated with levamisole phosphate (Ripercol L 150 F® – Fort Dodge, Brazil) and albendazole (Valbazen 10 Cobalto® – Pfizer, Brazil) for three consecutive days. Subsequently, the animals also received trichlorfon (Neguvon® – Bayer, Brazil) for an additional three days. Seven days later, fecal examinations were repeated and three animals that were still shedding eggs in faeces were subjected to a new series of treatment with the same anthelmintics. Nematode trichostrongylid eggs were not detected in the fecal tests after this procedure.

Afterwards, the 30 lambs were distributed into 10 classes of three animals each according to their live weight. One animal from each class was randomly allocated to one of the following groups: (1) infected group – artificially infected with *T. colubriformis* and fed *ad libitum*; (2) pair-fed group – non-infected and fed with the same amount of food consumed by the infected animals of the same class on the previous day; and (3) control group – non-infected and fed *ad libitum*.

During all the experimental period the lambs received *ad libitum Cynodon dactylon* (Tifton 85) hay (7% crude protein and 43 total digestible nutrients) and a commercial concentrate (18% crude protein) at 2% live weight of the lambs, Deccox® (Alpharma, Australia) was administered together with the concentrate, according to the manufacturer's instructions to prevent against coccidiosis.

2.2. Details about artificial infections

The lambs were orally infected with 2500 *T. colubriformis* infective larvae (L3), three times every week (Mondays, Wednesdays and Fridays) during 13 weeks; thus, each lamb of the infected group received a total of 97,500 L3. The infective larvae used in this experiment were from a *T. colubriformis* isolate obtained from sheep in 2003 (Rocha et al., 2007), and kept frozen in liquid nitrogen (MAFF, 1986) until used to infect two donor lambs. Fecal samples from these donor animals were collected weekly into collection bags for the production of infective larvae in fecal cultures (Ueno and Gonçalves, 1998).

2.3. Measurements

Analyses were performed for all animals, with the exception of histological and immunological analyses that were not carried out for samples from the pair-fed lambs.

2.3.1. Performance of lambs and food intake
The lambs were weekly weighed and food intake was measured daily. Food conversion rate of each animal was

calculated, based on the following formula: food conversion rate total = total food intake during 12 weeks of trial/total weight gain during 12 weeks of trial. The animals underwent a solid and liquid fast for 24 h, starting before the necropsies, thus, they were not weighed at the 13th week.

2.3.2. Fecal examination, hematology and biochemistry

Individual fecal and blood samples were collected weekly from the animals. Fecal egg counts (FEC) and composite fecal cultures were carried out for each group according to Ueno and Gonçalves (1998). Composite fecal cultures were prepared for each experimental group in order to confirm the monospecific infection by *T. colubriformis* in the infected group and the absence of nematode infections in the pair-fed and control groups. Third stage larvae (L3) were identified, according to Ueno and Gonçalves (1998).

Blood samples were collected directly from the jugular vein into vacutainer tubes with and without anticoagulant (EDTA). Packed cell volume (PVC) was determined through centrifugation in microhematocrit tubes. Eosinophils were quantified in a Neubauer chamber after staining with Carpentier's solution (Dawkins et al., 1989).

The blood in the tube without anticoagulant was centrifuged to allow serum separation. Subsequently, aliquots of serum samples were stored at −20 °C. In the same serum samples, the concentrations of total protein (g/100 mL) and albumin (g/100 mL) were determined using the techniques of biuret and bromocresol green (Protal método colorimétrico® – Laborlab, Brazil), respectively. The serum samples were applied in duplicate to the microplates in a volume of 200 μL per well and their absorbance was read at 562 nm and 625 nm, respectively, in an automated ELISA reader (Amersham-Biosciences, UK). The albumin/globulin ratio was assessed, based on the following formula: albumin/globulins ratio = albumin concentration/(total protein concentration − albumin concentration).

2.3.3. Worm burden determination and cranial duodenal lymph node weighing

After euthanasia, the small intestines were removed, opened and the contents were collected in graduated buckets. The intestines were then subjected to digestion in saline solution for 4 h at 38 °C to recover the nematodes present in the mucosa (Ueno and Gonçalves, 1998). Aliquots of 10% of the total intestine content and all the sediment of the material obtained in the digestion were collected, stored in plastic flasks and preserved with 5% formaldehyde. All nematodes present in the preserved material were quantified and identified, according to their developmental stage (Ueno and Gonçalves, 1998). During removal of the small intestine, the cranial duodenal lymph node of all animals from the infected and control groups were removed and weighed.

2.3.4. Histological analysis and scanning electron microscopy

Two tissue samples were collected from the small intestine of each animal and were fixed with buffered neutral formalin at 4% for 6 h. The first was a duodenal tissue sample collected at 10 cm from the pylorus and the second was a jejunal tissue sample taken at 1 m. Both samples were embedded in paraffin and processed according to routine histological techniques.

Eosinophil and mast cells were enumerated in 5 μm sections stained with hematoxylin–eosin or toluidine blue (Sigma–Aldrich, USA), respectively. Globular leukocytes were quantified in hematoxylin–eosin-stained sections under ultraviolet light. Cells were counted in 30 random fields from the muscular layer to the mucosa surface. The results of cell counts were expressed as arithmetic mean of cell number/mm^2 of mucosa.

Changes in the surface of the duodenal villous were analyzed using scanning electron microscopy in tissue samples collected from two animals that, throughout the experiment, presented the highest FEC, as well as from two randomly selected animals of the control group. The collected material was fixed with 2.5% glutaraldehyde diluted in phosphate buffer (pH 7.3–0.1 M) for 48 h, then processed using the routine techniques for scanning electron microscopy.

2.3.5. Enzyme-linked immunosorbent assay

Immunoglobulin A (IgA) and immunoglobulin G (IgG) levels against the total L3 and adult *T. colubriformis* antigens were assessed in serum samples by ELISA. IgA levels were also assessed against the same antigens in the intestinal mucus.

2.3.5.1. Antigen preparation.
To prepare antigens, infective larvae were produced in fecal cultures with the faeces of donor lambs monospecifically infected with *T. colubriformis*. These same animals were then sacrificed for the recovery of adult parasites, which were also used for antigen preparation.

The infective larvae were exsheathed (MAFF, 1986) and kept refrigerated in one single Falcon tube, which was subjected to centrifugation. Supernatant was removed and a small ultra pure water volume, sufficient to cover the larvae, was left. This tube received 2 mL phosphate buffer (PBS) at 4 °C, supplemented with protease inhibitor (Complete-Mini® – Roche, USA). L3 were fragmented using an ultrasonic processor (Vibra-Cell® – Sonics & Materials Inc., USA) in 20 cycles of 1 min at 2 min intervals to avoid heating. To extract soluble proteins, the material was then centrifuged for 30 min, at 15,000 × g and 4 °C. Supernatant was collected, separated into aliquots and stored in a freezer at −80 °C.

Adult specimens of *T. colubriformis*, obtained from infected animals, were washed five times in PBS (pH 7.2, 4 °C) and placed in a tube containing 2 mL PBS, at 4 °C, supplemented with protease inhibitor (Complete-Mini®, Roche, USA). Adult parasites were fragmented using an Ultra Turrax® (Ika, Germany). The extract was centrifuged (15,000 × g) at 4 °C for 20 min and the supernatant containing the adult-soluble-antigen extract was collected and frozen at −80 °C until further use.

Total protein concentrations of L3 and adult antigens were determined using a kit (Protal método colorimétrico® – Laborlab, Brazil) and absorbance was read at 560 nm

using a spectrophotometer (Ultrospec 2100 pro® – Amersham Pharmacia Biotech, England).

2.3.5.2. Parasite-specific serum IgG and IgA. In 96-well microplates (F96 MicroWell plate – Maxisorp®, NUNC, USA), larval and adult *T. colubriformis* crude antigens, at a concentration of 2 μg/mL, were incubated with carbonate buffer pH 9.6, overnight (16 h) at room temperature, in a volume of 100 μL per well. After incubation, microplates were washed three times in an automated washing machine (ELx405® – BioTek, USA) with a solution constituted of ultra pure water and 0.05% Tween 20 (Pro Pure® – Amresco, USA). Following this step, microplates were incubated for 1 h at 37 °C with 100 μL per well of PBS–GT blocking buffer (pH 7), with 0.1% Gelatin (Amresco, USA) and 0.05% Tween 20 (Amresco, USA). Microplates were again washed with washing solution and diluted serum samples were added. Serum samples were diluted with PBS–GT at 1:2000 for IgG and at 1:500 for IgA measurement and applied in duplicate to the microplates in a volume of 100 μL per well. Plates were again incubated for 1 h at 37 °C.

For IgG determination, samples were then incubated for 1 h at 37 °C with rabbit polyclonal to sheep IgG (Abcam; 1:1000 in PBS–GT) followed by polyclonal goat anti-rabbit immunoglobulins linked to alkaline phosphatase (Dako, Denmark; 1:4000 in PBS–GT). For IgA determination, incubations were carried out using monoclonal mouse anti-bovine/ovine IgA antibody (Serotec; 1:250 in PBS–GT), followed by polyclonal goat anti-mouse conjugate, linked to alkaline phosphatase (DAKO, Denmark; 1:1000 in PBS–GT).

After the last wash, 100 μL of PNPP (p-nitrophenylphosphate, PNPP kit® – Pierce, USA) substrate was added to each well and the enzymatic reaction was developed for 30 min at room temperature and 90 min at 4 °C. Plates were then read on an ELISA reader (Amersham-Biosciences), at 405 nm optical density, and the results were expressed as the percentage of optical density value (OD), using the serum of a positive animal as a reference (Kanobana et al., 2001) and employing the following formula: % OD = [(OD mean of the tested serum – OD mean of blank)/(OD mean of the positive standard serum – OD mean of blank)] × 100.

2.3.5.3. Parasite-specific IgA in the intestinal mucous. At necropsy, two mucus samples were collected from a segment of the small intestine, located between 10 cm and 20 cm from the pylorus. The segments were opened and the mucosa surface was scraped with a glass slide. The sample was placed in a 50 mL Falcon tube to which were added 3 mL PBS, supplemented with protease inhibitor (1 tablet of Complete®, Roche in 25 mL PBS pH 7.0).

Samples were homogenized for 1 h at 4 °C. Following this step, tubes were centrifuged (3000 × g) for 30 min at 4 °C. Supernatant was removed and centrifuged again (15,000 × g) for 30 min, at 4 °C, separated into aliquots and stored at −20 °C (Kanobana et al., 2002). Protein concentration was assessed through the biuret technique (Protal método colorimétrico® – Laborlab) and absorbance was read with a 562 nm filter using an automated
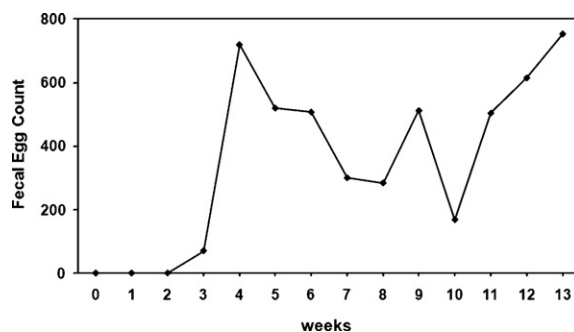

Fig. 1. Mean back-transformed $\log_{10}$ fecal egg counts (FEC) of *Trichostrongylus colubriformis* in Santa Ines lambs of the group infected three times per week with 2500 larvae of de *T. colubriformis* and fed *ad libitum*.

microplate spectrophotometer (Amersham–Biosciences). Supernatant samples were adjusted to a final concentration of 8 mg protein/mL, and ELISA reactions for IgA against L3 and against adult *T. colubriformis* were as previously described for serum analysis with 1/25 mucus dilution. Results were expressed in OD of sample minus OD of blank (Kanobana et al., 2001).

2.4. Experimental design and statistical analysis

The significant differences between variables of the groups were assessed by oneway analysis of variance using the statistical software Minitab® (version 11.21). Group means were compared using the Tukey's test, at the 1% and 5% significance level. The weekly variables were analyzed with general linear model of the repeated measures for statistical software SPSS® (version 17.0), considering the experimental groups as between-subjects factor and time as within-subjects factor. According to result found in the assumption test of sphericity, Huynh-Feldt or Greenhouse Geisser corrections were used for the analysis of the major interaction effects, at the 1% significance level.

Results of normal data were expressed as arithmetic means (±standard error). The data relative to FEC, worm burden, blood eosinophils, inflammatory cell counts and immunoglobulins levels were previously $\log_{10}(x+1)$ transformed to stabilize the variance before the analysis (non-normal data), however, results were expressed as back-transformed means for easier interpretation.

3. Results

3.1. Fecal examination, worm burden and clinical signs of infection

T. colubriformis eggs were detected in the faeces of the infected group starting at the third week post-infection with peaks in FEC mean back-transformed $\log_{10}$ at the fourth and 13th weeks post infection (Fig. 1). Eggs were not detected in control and pair-fed animals during the entire trial. The mean back-transformed $\log_{10}$ of *T. colubriformis* specimens, found in the infected group, was 6345.8. Six animals presented a low worm burden, ranging from 13 to 1540 parasites, i.e., <1.6% of the administered infective larvae were capable of establishing. In contrast, four

animals presented more than 6000 adult parasites. The highest worm burden was 26,830 *T. colubriformis* adult specimens, which corresponded to the establishment of 27.5% of the infective larvae. None of the infected animals presented immature stages of the parasite in the analyzed material.

At the ninth week after the beginning of infections, eight lambs from the infected group showed alterations in faeces, eliminating agglomerated pellets with a "grape bunch" aspect, which had a variable consistency from semi-solid to pasty and contained intestinal mucus. At the 10th week post-infection, the other two lambs of the infected group also started eliminating faeces with the above-mentioned characteristics. This alteration persisted in all individuals of the infected group until the end of the trial. Conversely, control and pair-fed animals had faeces of normal consistency. Clinical signs such as apathy, weakness and discomfort were also observed in two animals infected at the ninth week post-infection and in other lamb at the 11th week post-infection. These symptoms lasted for one week in each animal and these lambs were those that showed the lowest worm burden at the end of the trial.

3.2. Performance of the animals and food intake

The infected group presented the lowest live weight means, starting at the sixth week post infection. However, there was no statistical difference ($P > 0.05$) between group means. There was a highly significant live weight × time interaction ($P < 0.001$). The initial means of live weight and the means at 12 weeks post infection were, respectively: 20.44 ± 1.53 kg and 29.45 ± 2.30 kg (infected group); 20.65 ± 1.25 kg and 32.11 ± 1.99 kg (pair-fed group) and 20.20 ± 1.59 kg and 34.57 ± 2.18 kg (control group). The daily mean weight gain of the infected (107.26 ± 10.8 g/day) and pair-fed (136.43 ± 9.86 g/day) groups were significantly lower ($P < 0.01$) than the mean of the control group (171.07 ± 7.15 g/day).

The concentrate supplied to the lambs was totally consumed during the experimental period. However, there were differences between groups concerning daily mean voluntary hay food intake. The infected group presented a lower voluntary hay food intake than the control group throughout the experiment, but this difference was significant statistically ($P < 0.01$) only at the ninth and 12th weeks post-infection. On these occasions, the control group consumed 798.50 ± 42.60 g and 837.86 ± 46.10 g, while the infected group ingested 605.45 ± 62.10 g and 677.44 ± 50.30 g, 24% and 19% of the reduction, respectively. There was no significant voluntary hay food intake × time interaction ($P = 0.049$). The infected group had the worst food conversion rate, in comparison with the other two groups ($P < 0.01$). The animals from this group needed to consume on average 10.05 ± 0.52 kg of food to gain 1 kg of live weight, whereas the pair-fed and control groups consumed, respectively, 7.82 ± 0.41 kg and 6.85 ± 0.17 kg of food to gain this same live weight.

In the infected group, four animals demonstrated more than 6000 adult parasites (sub-group with high worm burden) and six animals had <1600 *T. colubriformis* adult specimens (sub-group with low worm burden). These sub-groups had, respectively, daily mean weight gains of 117.56 ± 16.80 g/day and 100.40 ± 14.60 g/day ($P > 0.05$). The food conversion rate of the susceptible (high worm burden) and resistant (low worm burden) sub-groups were, respectively, 9.65 ± 0.68 kg and 10.31 ± 0.77 of consumed food to gain 1 kg live weight ($P > 0.05$), i.e., the performances of both infected sub-groups were similar.

3.3. Hematology and biochemistry

The PCV mean of the infected group ($29.80 \pm 0.49\%$) was significantly lower ($P < 0.05$) than the PCV of the other two groups (control group = $32.70 \pm 0.79\%$; pair-fed group = $32.30 \pm 0.76\%$), only in the last experimental week. There was no significant PVC × time interaction ($P = 0.022$).

As regards total serum protein, the infected group presented statistically lower ($P < 0.01$) means (week 8 = 5.69 ± 0.14 g/100 mL; week 9 = 5.92 ± 0.12 g/100 mL; week 10 = 5.61 ± 0.12 g/100 mL; week 11 = 6.24 ± 0.12 g/100 mL; week 12 = 5.66 ± 0.14 g/100 mL; week 13 = 6.36 ± 0.12 g/100 mL), compared with those of the control group (week 10 = 6.21 ± 0.11 g/100 mL; week 11 = 6.84 ± 0.15 g/100 mL; week 12 = 6.12 ± 0.09 g/100 mL) and the pair-fed group (week 8 = 6.39 ± 0.08 g/100 mL; week 9 = 6.54 ± 0.13 g/100 mL; week 10 = 6.37 ± 0.10 g/100 mL; week 11 = 6.96 ± 0.17 g/100 mL; week 12 = 6.39 ± 0.11 g/100 mL; week 13 = 7.02 ± 0.13 g/100 mL), respectively, from the 10th to the 12th week and from the eighth to the 13th week post-infection. The pair-fed group had a statistically higher ($P < 0.05$) total serum protein mean (6.86 ± 0.09 g/100 mL) than the control group (6.35 ± 0.12 g/100 mL) only in the second week. There was no total serum protein × time interaction ($P = 0.203$).

Mean values of albumin serum concentrations were significantly lower in the infected group (week 6 = 2.99 ± 0.08 g/100 mL; week 8 = 2.40 ± 0.12 g/100 mL; week 9 = 2.59 ± 0.09 g/100 mL; week 10 = 2.28 ± 0.08 g/100 mL; week 11 = 2.76 ± 0.08 g/100 mL; week 12 = 2.68 ± 0.08 g/100 mL; week 13 = 2.82 ± 0.44 g/100 mL) than control
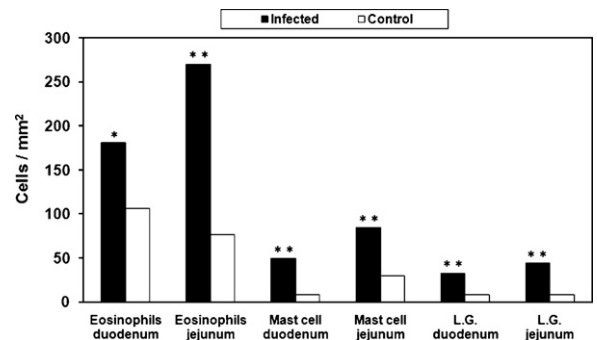

Fig. 2. Mean back-transformed $\log_{10}$ values of eosinophils, mast cells and globular leukocytes (G.L.) per mm^2 of the duodenal and jejunal mucosa in Santa Ines lambs infected three times per week with 2500 larvae of *Trichostrongylus colubriformis* and fed *ad libitum* (Infected); and non-infected group fed *ad libitum* (control). The symbols * and ** stand for significant difference, with $P < 0.05$ and $P < 0.01$, respectively.
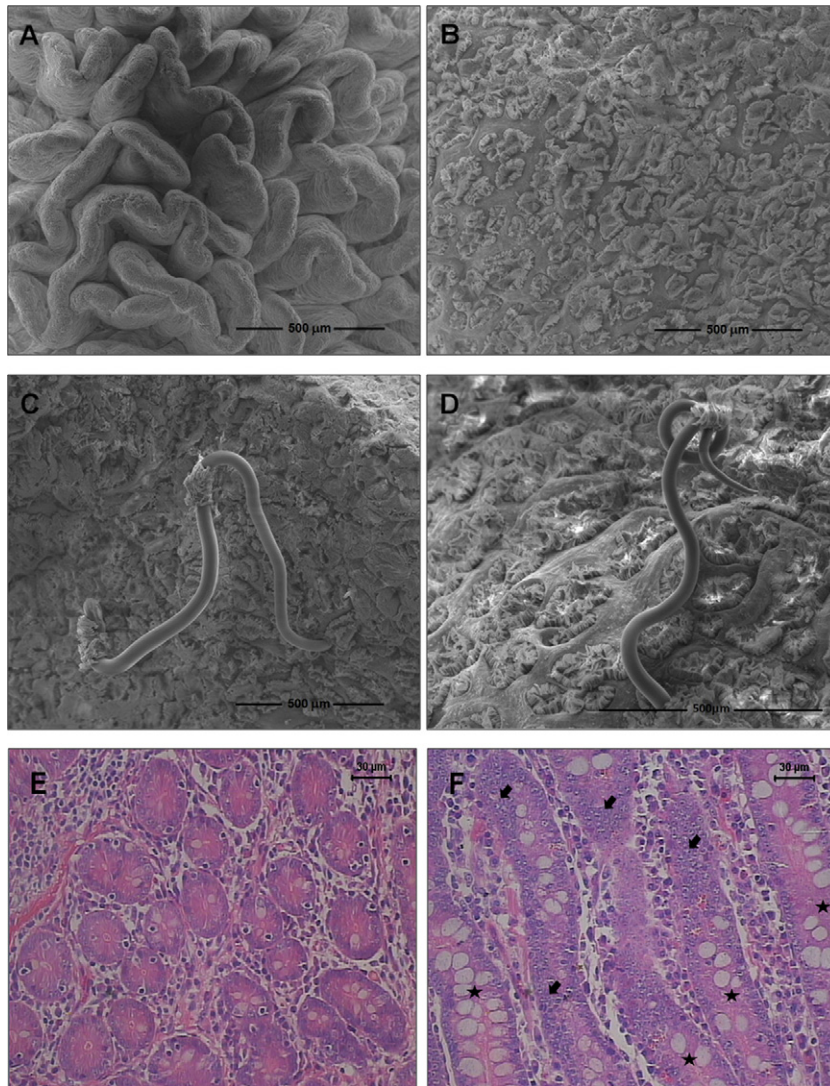
Fig. 3. Villous of duodenal mucosa in a lamb of the control group, showing normal morphology (A); generalized duodenal villous atrophy in a lamb of the infected group (B); male specimen of *T. colubriformis* inserted in the injured duodenal mucosa in lamb of the infected group (C); anterior extremity of *T. colubriformis* emerging from tunnels in an area with villous atrophy and erosion of the epithelium of duodenal mucosa (D); intestinal crypts in lamb of the control group, showing normal range (E); crypts hyperplasia and hypertrophy in lamb of the infected group, with increased number of goblet (★) and epithelial cells (→), the latter presenting overlapped nuclei (F).

group (week 6 = 3.31 ± 0.07 g/100 mL; week 8 = 2.86 ± 0.16 g/100 mL; week 9 = 3.26 ± 0.08 g/100 mL; week 10 = 2.97 ± 0.13 g/100 mL; week 11 = 3.43 ± 0.10 g/100 mL; week 12 = 3.50 ± 0.07 g/100 mL; week 13 = 3.59 ± 0.37 g/100 mL) and pair-fed group (week 6 = 3.27 ± 0.09 g/100 mL; week 8 = 2.90 ± 0.06 g/100 mL; week 9 = 3.29 ± 0.08 g/100 mL; week 10 = 2.98 ± 0.08 g/100 mL; week 11 = 3.55 ± 0.13 g/100 mL; week 12 = 3.45 ± 0.10 g/100 mL; week 13 = 3.43 ± 0.30 g/100 mL) in the sixth and eighth ($P < 0.05$) and from the ninth to the 13th week post-infection ($P < 0.01$). In the sixth week, the albumin serum concentrations of the control group were significantly higher ($P < 0.05$) than the pair-fed group. There was no albumin serum concentrations × time interaction ($P = 0.002$).

With regard to the albumin/globulins ratio, the infected group (week 6 = 0.94 ± 0.06; week 7 = 1.07 ± 0.09; week 9 = 0.78 ± 0.03; week 10 = 0.69 ± 0.04; week 11 = 0.80 ± 0.03; week 12 = 0.93 ± 0.07; week 13 = 0.82 ± 0.06) had significantly lower values than those of the pair-fed group (week 7 = 1.41 ± 0.11; week 9 = 1.03 ± 0.06; week 10 = 0.90 ± 0.05; week 11 = 1.09 ± 0.11) in the seventh ($P < 0.05$), ninth, 10th ($P < 0.01$) and 11th ($P < 0.05$) weeks post-infection, and relative to the control group (week 6 = 1.16 ± 0.06; week 9 = 1.12 ± 0.06; week 10 = 0.95 ± 0.08; week 12 = 1.37 ± 0.09; week 13 = 1.15 ± 0.05) in the sixth ($P < 0.05$), ninth, 10th, 12th and 13th ($P < 0.01$) weeks post-infection. The control group (0.82 ± 0.05) had a significantly higher ($P < 0.05$) albumin/globulins ratio than the pair-fed group (0.67 ± 0.02) in the fourth week.
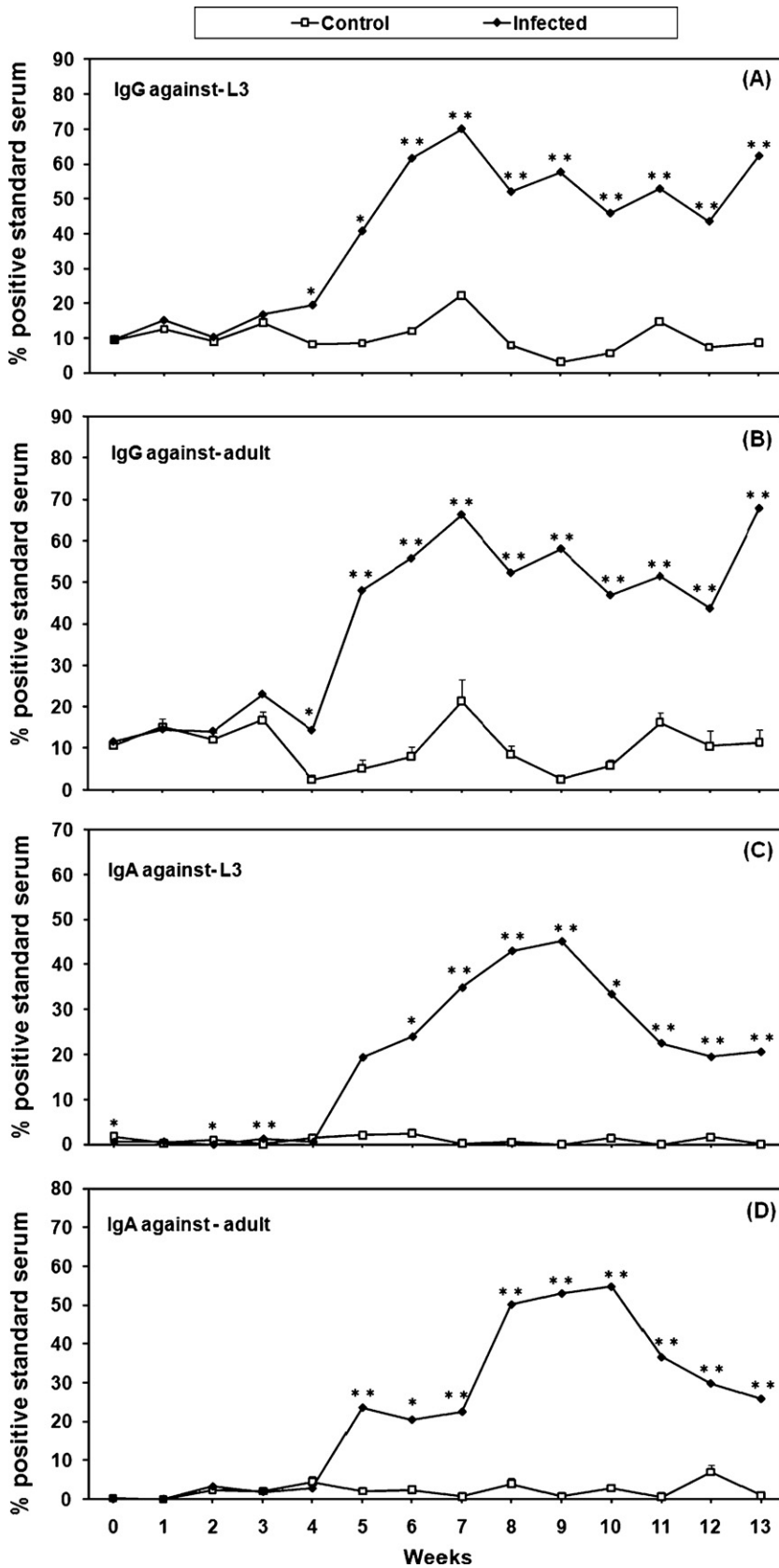
Fig. 4.

There was no albumin/globulins ratio × time interaction ($P = 0.017$).

3.4. Blood eosinophils

The infected group demonstrated an increased mean blood eosinophil number (week 8 = 935.00 cells/μL; week 11 = 1105.00 cells/μL; week 13 = 1292.50 cells/μL), which was significantly higher than that of the control group (week 8 = 140.00 cells/μL; week 11 = 240.00 cells/μL; week 13 = 65.00 cells/μL) on the eighth ($P < 0.01$), 11th ($P < 0.05$) and 13th ($P < 0.01$) weeks post-infection. There was a highly significant blood eosinophil number × time interaction ($P < 0.001$).

3.5. Inflammatory cells in the mucosa and weight of duodenal cranial lymph node

The mean number of eosinophils, mast cells and globular leukocytes in duodenum and jejunum mucosa of the infected group was significantly higher, compared with the control group (Fig. 2). The mean weight of the duodenal cranial lymph node was also significantly higher ($P < 0.01$) in the infected group (1.79 ± 0.80 g) than that of the control group (0.89 ± 0.33 g).

3.6. Pathological changes in the duodenal mucosa

Scanning electron microscopy and histopathology showed severe pathological changes on the surface of the duodenal mucosa of the two infected animals that were analyzed (Fig. 3). The alterations observed were; generalized villous atrophy, including formation of tunnels in the duodenal epithelium; erosion of the epithelium; hyperplasia and hypertrophy of the intestinal crypts, with increased number of goblet and epithelial cells, the latter presenting overlapped nucleus; hemorrhagic areas and inflammatory infiltrate with predominance of mononuclear leukocytes.

3.7. Serum and intestinal mucus antibody

The infected group had significantly higher specific serum levels of IgG against L3 of T. colubriformis than those of the control group in the fourth and fifth weeks post-infection ($P < 0.05$), and this difference was highly significant ($P < 0.01$) in the sixth to 13th weeks post-infection (Fig. 4). Mean concentrations of IgG against adult antigens of T. colubriformis were significantly higher in the infected group in the fourth week ($P < 0.05$) and highly significant from the fifth to the 13th ($P < 0.01$) weeks post-infection (Fig. 4). Highly significant interactions were observed for the specific serum levels of IgG against L3 of T. colubriformis × time interaction ($P < 0.001$) and specific serum levels of IgG against adult of T. colubriformis × time interaction ($P < 0.001$).

The infected lambs also had significantly higher serum levels of IgA against L3 ($P < 0.05$) than the control animals in the sixth and 10th weeks post-infection, and this difference was highly significant ($P < 0.01$) in the third, from the seventh to ninth, and from the 11th to the 13th weeks post-infection (Fig. 4). Only in weeks zero and two did the control group have statistically higher serum levels of IgA against L3 ($P < 0.05$) than the infected group. As regards IgA against adult T. colubriformis, the infected group presented significantly higher means than the control group in the sixth week post-infection ($P < 0.05$), and these differences were highly significant ($P < 0.01$) in the fifth and from the seventh to the 13th week post-infection (Fig. 4). Highly significant interactions were observed for the specific serum levels of IgA against L3 of T. colubriformis × time interaction ($P < 0.001$) and specific serum levels of IgA against adult of T. colubriformis × time interaction ($P < 0.001$). The levels of IgA against L3 and against adult T. colubriformis in the intestinal mucus of the infected group (OD = 0.364 and 0.392) were significantly higher ($P < 0.05$ and $P < 0.01$, respectively), compared with the control group (OD = 0.03 and 0.02).

4. Discussion

There was a marked variation in worm burden amongst animals. Most of the lambs had few parasites: 13–1540 nematodes in six animals, representing an establishment of <1.6% of the inoculum, whereas four lambs had a relatively high parasitic load, of 6310–26830 adults specimens. Similar variability was found in male Santa Ines sheep, aged approximately one year and those naturally infected with gastrointestinal nematodes, which also showed an aggregated distribution of parasites with a mean of 4897 T. colubriformis specimens and worm burden ranging from 290 to 31,300 parasites (Amarante et al., 2007). According to Dobson et al. (1990a), the variability between host worm burdens increases over the course of infection and the primary mechanism for T. colubriformis adult worm elimination is the rejection by the host.

However, Santa Ines lambs, subjected to only one artificial infection with 4000 T. colubriformis larvae, had an average of 1473 parasites 40 days after infection, i.e., 36.8% of the administered larvae established as adult nematodes (Almeida et al., 2010), this establishment percentage was much higher than that recorded for the animals of the present study, indicating that the immune response of infected lambs, acquired through continuous challenge, caused an extensive reduction in the worm burden of most of the animals. According to Dobson et al. (1990a,b,c), the primary manifestation of host immunity against continuous infection for T. colubriformis is a reduction in the number of incoming larvae that became established, this is followed by arrested worm development, reduced worm fecundity and the eventual loss of the established worm

Fig. 4. Mean back-transformed $\log_{10}$ levels of serum IgG against third stage larvae (L3) (A), IgG against adult (B), IgA against L3 (C) and IgA against adult (D) antigens of Trichostrongylus colubriformis in Santa Ines lambs of infected and control groups. The symbols * and ** stand for significant difference, with $P < 0.05$ and $P < 0.01$, respectively.

population; this response is influenced by rate and duration of infection, and by host age.

Several studies have demonstrated the central role of the acquired immune response in the resistance against gastrointestinal nematode infections in sheep (Peña et al., 2004; Shakya et al., 2009). This response has been associated with the activity of Th2CD4^{+} lymphocytes, eosinophilia and increased number of inflammatory cells in the mucosa such as eosinophils, mast cells and globular leukocytes (Amarante and Amarante, 2003). As reported by Pernthaner et al. (2006), another immunological process was also observed, where high levels of specific immunoglobulins (IgG and IgA) against *T. colubriformis* larvae and adults were detected. In animals of the present study, an immune response of this type was evident and prevented most of the infective larvae from establishing themselves as adults.

Paradoxically, the highest worm burden, 26,830 specimens (27.5% of the inoculum), was recorded in one animal that, at the end of the experiment, presented the highest antibody levels in the blood and mucus and the lowest eosinophils and mast cells counts in the mucosa of the small intestine. These findings emphasize the importance of the inflammatory cells in the mucosa and also indicate that the efficiency of the immune response depends not only on the presence or quantity of the immunological components, but also on the interaction of these elements together.

Starting at the ninth or 10th weeks post infection until the end of the trial, all Santa Ines lambs presented faeces with an altered aspect and consistency, however clinical signs of severe diarrhea were not observed in these animals. In the three Santa Ines lambs with the lowest worm burdens, in addition to the alterations in their faeces, they also presented clinical signs of apathy, weakness and discomfort, during the ninth or 10th week post infection. These alterations were probably caused by the severe immunopathological changes in the intestinal mucosa that occurred as a consequence of the constant contact with infective larvae, during their attempt to establish in the mucosa.

In New Zealand, Morris et al. (2000) observed in Romney sheep, selected for low FEC (resistant) and infected naturally by *Trichostrongylus* spp. and *Ostertagia* spp., a productive performance that was lower than that displayed by sheep selected for high FEC. The resistant animals had the lowest mean weight gain and wool production and also had a higher incidence of diarrhea, with a greater accumulation of faeces around the fleece breech. Similarly, in Australia, severe diarrhea with accumulations of faeces around the fleece breech was observed in Merino ewes selected for low FEC and naturally infected with the same parasites (Larsen et al., 1994, 1999). This phenomenon can be explained by changes in cellular response, including: significantly more eosinophils in the gut mucosa, changes in lymphocyte, reduced CD8^{+} cells, increase in the ratio of cells CD4^{+}:CD8^{+}, and reduced reactive cells to interferon gamma, compared to animals free of the problem (Larsen et al., 1994, 1999).

PCV values were slightly decreased during the last weeks of the trial in infected animals, probably as a consequence of the hemorrhagic lesions in intestinal tissues, as observed from histological analyses. Alterations in PCV values are more common in parasitism by hematophagous nematodes, such as *H. contortus* (Shakya et al., 2009). The major alteration in blood variables occurred in the total plasma protein and albumin serum concentrations, especially during the second half of the trial. The rejection of *T. colubriformis* incoming larvae by immune sheep is accompanied by an intestinal inflammatory response involving the secretion of biogenic amines with a concurrent plasma loss. This is the major factor responsible for the development of hypoproteinemia and hypoalbuminaemia in lambs infected with *T. colubriformis* (Steel et al., 1980). In the present study, the Santa Ines lambs with the highest worm burdens also presented the lowest albumin serum concentrations at the end of the trial. The increase in the globulins concentrations in the infected group was observed in the second half of the present trial, characterized by the lowest albumin/globulins ratio, which coincided with the period with the highest levels of all analyzed immunoglobulins. These increases may be associated with the development of immunity, i.e., there was a rise in the synthesis of immunoglobulins as a consequence of the infections, as suggested by Steel et al. (1980).

The depression in appetite was small and not the main disorder caused by the parasitism in Santa Ines lambs. Nevertheless, the infected group had the lowest voluntary hay food intake, when compared with the control group during the trial; however, this difference was statistically significant only at two weeks. Severe consumption disorders have been previously reported in young lambs infected with a large number of larvae (Steel et al., 1980; Symons, 1983). Symons and Hennessy (1981) demonstrated elevated levels of the cholecystokinin (CCK) in sheep infected with *T. colubriformis* and concluded that the reduced appetite of animals infected with this nematode may be due to, or mediated by, an increase in the plasma concentration of CCK and that the parasites stimulate secretion of this hormone.

It is possible that some pro-inflammatory cytokines involved in cell signaling of the non-specific acute phase response, such as IL-1, IL-6, IL-8, IFN-γ and TNF-α, may act in the hypothalamus, also causing a disturbance in feeding behavior (Johnson, 1997; Spurlock, 1997; Langhans, 2000). In a study with wool sheep, voluntary food intake was not significantly affected by challenge with *T. colubriformis* in experienced immunologically resistant mature sheep, while the effect of parasitic infection was much more pronounced in young naïve lambs, which presented a temporary reduction in food intake, occurring only during the phase of acquisition of immunity, between days 14 and 64 of infection and coinciding with the period of elevation of serum IgA against L3 larvae (Greer et al., 2005).

In agreement with several studies in wool sheep (Barker, 1973a,b, 1975a,b), the young Santa Ines hair sheep, infected with *T. colubriformis*, also showed severe lesions in the small intestine mucosa, such as generalized villous atrophy and erosion in the duodenal epithelium. These lesions would likely cause a decrease in the efficiency of nutrient digestion and absorption, causing significant impairment in their productive performance. This hypothesis is reinforced when the food conversion rate of the infected and pair-fed groups are compared, i.e., these

variables were better in the latter, although the food quantity provided was the same for both groups. The reductions of 37% and 30% in daily mean weight gain, beyond the increase of 46% in the food conversion rate were observed in the infected group, when compared with the control group. Greer et al. (2005) also found a similar proportional decrease of 30% in live weight gain in wool lambs infected with *T. colubriformis*.

Independently of the worm burden, all infected animals had a similar performance. Therefore, the reduction in productive indices of the infected group were due not only to the direct damages caused by adult nematodes per se in intestinal mucosa, but possibly due to the high nutrient and energy demand diverted to the acquisition and maintenance of immunity against larvae and/or repair of the damaged intestinal tissues. Sykes (1994) observed that the maintenance of immune response against parasitic nematodes in sheep may cause a 15% loss in productivity. According to Kyriazakis and Houdijk (2006), the metabolizable protein requirement increases by 20–25% in growing lambs infected by gastrointestinal nematodes, as the components of the immune response such as immunoglobulins, leukotrienes, eosinophils, mast cells, globule leucocytes and cytokines and intestinal tissue cells are composed primarily of protein. The gastrointestinal tract is a highly competitive tissue, requiring large quantities of amino acids to perform its functions and the amino acid demand increases during subclinical infections with *T. colubriformis* due to the need for repairing damaged tissues. This impairs the amino acid intake in other tissues, consequently decreasing the sheep's anabolism, leading to negative effects in productive indices (Yu et al., 2000). In all infected lambs of the present study, the repairing damaged tissues were characterized by hyperplasia and hypertrophy of the intestinal crypts, with an increased number of young epithelial cells and overlapped cellular nuclei.

Greer et al. (2005, 2008) observed the absence of effects on the performance of the naïve lambs infected with *T. colubriformis* and *T. cincumcincta*, when animals were immunosuppressed with glucocorticoid methylprednisolone acetate, despite the high mean worm burdens of more than 70,000 and 40,000 parasites, respectively. In these studies, the infected lambs, but not the immunosuppressed lambs, presented a reduction of 20% in efficiency of metabolizable energy utilization, in comparison with animals infected with the same nematodes but that were immunosuppressed. Further studies should be conducted to better understand the impact of several immunological effects on the performance of Santa Ines lambs infected by *T. colubriformis*.

One of the measures employed to avoid the development of anthelmintic resistance is the use of selective treatments. In areas of occurrence of *H. contortus* infections, FAMACHA is the most often employed method, which suggests treating only anemic animals, besides keeping the flock in pastures contaminated with nematode infective larvae, in order to preserve the population "*in refugia*" (Van Wyk and Bath, 2002). More detailed studies on the economic impact of this method are needed since it disregards the effects of *T. colubriformis* parasitism in young sheep, as observed in the present study. Therefore, when selective treatment is adopted, other variables should also be considered in the identification of animals that need anthelmintic treatment, such as weight gain and changes in fecal consistency.

In conclusion, the negative impact of *T. colubriformis* infection on the productive performance of the Santa Ines lambs can be considered high and may compromise the profitability and commercial viability of sheep husbandry in Brazilian farms, where prophylaxis of this species is neglected. Santa Ines young sheep should be kept in grazing areas with low contamination by infective larvae of *T. colubriformis*. Another option would be the selection of resistant animals that eliminate small quantities of eggs in their faeces, consequently decreasing pasture contamination with infective larvae. The low number of infective larvae in the environment would lead to a significant decrease in the exposure of animals to infections. It should be emphasized that selecting resistant sheep to *T. colubriformis* should not be based only on parasitological parameters, such as FEC and worm burden, but also on the production performance and lack of clinical signs of parasitic gastroenteritis.

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