Veterinary Parasitology 311 (2022) 109811

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Effect of three feeding levels on the pathogenesis and establishment of *Haemonchus contortus* in parasite-naïve Pelibuey hair sheep lambs during their first infection

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ARTICLE INFO

Keywords:
Immunoglobulin G
Host resistance
Host resilience
Feeding level

ABSTRACT

This study evaluated the effect of three feeding levels on the pathogenesis and establishment of *H. contortus* upon the first infection of parasite-naïve Pelibuey hair sheep lambs. Forty-two 6-month-old hair sheep lambs (24 ± 4 kg) raised parasite free from birth were used. The lambs were assigned to 3 groups (n = 14), and each was fed a diet designed for different daily weight gain (DWG): 75 g/d (Diet 1), 125 g/d (Diet 2) and 200 g/d (Diet 3). After four weeks of diet adaptation, 10 lambs/group were infected with 450 L_3 *H. contortus*/kg BW (infected), and 4 lambs/group were kept parasite-free (NInf). DWG, hematocrit (Ht), hemoglobin (Hb), peripheral eosinophils (EOS), IgG concentration against *H. contortus*, and eggs per gram (EPG) of feces were measured in each lamb from day 14 before infection until day 29 postinfection (PI). On day 29 PI, the lambs were slaughtered to determine the total number of adult parasites (TAW), the length of the female worms, and the number of eggs *in utero* (EIU). Each group reached the expected DWG ($P = 0.001$), and there was no effect of infection or the diet × infection interaction. Ht was lower in infected lambs than in NInf lambs, and this difference was significant for animals on Diets 1 and 2 ($P = 0.044$). From day 14 PI onward, Hb was lower in the infected lambs than in the NInf lambs ($P = 0.001$). Furthermore, compared with NInf lambs, the infected lambs had higher EOS from day 7 PI and higher IgG from day 14 PI. Neither EOS nor IgG were affected by diet. Lambs on Diet 3 had lower EPG during patency than those fed Diets 1 or 2 (days 25 and 28 PI; $P = 0.002$). Furthermore, lambs fed Diet 3 had lower TAW (Diet 1 vs 3 $P = 0.037$; Diet 2 vs 3 $P = 0.049$) and EIU ($P = 0.004$) than lambs fed Diet 1 or 2. Lambs were resilient to infection regardless of diet. Although EOS and IgG were higher in all infected animals than in

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https://doi.org/10.1016/j.vetpar.2022.109811
Received 5 May 2022; Received in revised form 26 September 2022; Accepted 5 October 2022
Available online 8 October 2022

Ninf animals, EPG, TAW and EIU decreased only in lambs fed Diet 3. Thus, a diet targeting a DWG of 200 g/d can significantly limit the establishment of *H. contortus* in Pelibuey lambs infected for the first time.

1. Introduction

Gastrointestinal nematode (GIN) infections are a major health problem for grazing small ruminants. Commercial anthelmintics are the most widely used method of control for these parasites, but in many flocks in tropical areas, GIN populations are resistant to one or more classes of these drugs (Herrera-Manzanilla et al., 2017; Sepúlveda-Vázquez et al., 2021). Given this prevalence of resistance, nutritional management has been proposed as an alternative method for sustainable GIN control on sheep farms (Hoste and Torres-Acosta, 2011). Several pen and field studies have shown that dietary manipulation can improve the resilience and, in some cases, resistance of lambs of different breeds to GIN (Holmes, 1993; Coop and Holmes, 1996; Coop and Kyriazakis, 1999; Torres-Acosta et al., 2012; Hoste et al., 2016). In these reviews, it was proposed that resistance was achieved by providing sufficient nutrients to meet not only the lambs' maintenance requirements but also the additional demand required to fight a GIN infection, including tissue repair and the production of mucus and cells for the immune response (Knox et al., 2006, Hoste et al., 2016). By contrast, studies using parasite-naïve Scottish Blackface lambs, Finn Dorset lambs or Hampshire lambs have shown that a higher feeding level, mainly with dietary protein, does not limit the establishment of *Haemonchus contortus* L_3 larvae in the abomasum during the first infection, as fecal egg counts and adult parasite numbers remain the same regardless of the level of dietary protein supplementation during parasite establishment (Abbott et al., 1985; Wallace et al., 1995, 1996). This paradigm was thought to apply to other sheep breeds, but evidence in recent decades has shown that hair sheep breeds such as Santa Ines, Barbados Blackbelly, Saint Croix, Florida Native, Pelibuey and Red Maasai have natural resistance to GIN infections (Amarante et al., 2009; Jacobs et al., 2016; Zvinorova et al., 2016; Estrada-Reyes et al., 2017). González et al. (2008, 2011) and Hernández et al. (2016, 2017) observed disparate innate immune responses to *H. contortus* infection between Canarian hair sheep (a short-haired breed similar to Pelibuey reared for meat) and Canarian sheep (a coarse wool sheep breed reared for milk). The authors concluded that $\gamma\delta+$ T cells, in association with eosinophils and mucosal IgA, may play a role in immunity against the larval and adult stages of *H. contortus* and give Canarian hair sheep lambs an advantage over Canarian lambs. The potential resistance of hair sheep breeds highlights the importance of studying the effect of dietary manipulation on the establishment of *H. contortus* using parasite-naïve lambs of such breeds. Hence, this study evaluated the effect of three feeding levels on the pathogenesis and establishment of *H. contortus* during the first infection of parasite-naïve Pelibuey hair sheep lambs.

2. Materials and methods

2.1. Location

The present study was conducted from January 2020 to January 2021 at the Faculty of Veterinary Medicine and Animal Husbandry of the Autonomous University of Yucatan (FMVZ-UADY) in Merida, Yucatan, Mexico (20°52'07.2" N and 89°37'24.5" W). The Bioethics Committee of FMVZ-UADY approved the study (license no. CB-CCBA-I-2020–001).

2.2. Experimental animals

Forty-two Pelibuey lambs with an age of approximately six months and an average body weight (BW) of 24 kg ± 4 kg were used. The animals were raised in total confinement conditions (pens with concrete floors) from birth until inclusion in the study to avoid any GIN infection. Prior to the experiment, the lambs were fed a complete diet to allow 100 g daily weight gain (DWG) based on the AFRC (1993). The diet contained Johnson grass (*Sorghum halepense*) hay, ground corn, soybean paste, molasses, minerals, and a commercial feed with 18% protein. Water was offered *ad libitum* and renewed daily.

2.3. Experimental management

The adaptation period lasted from day − 28 to day 0 (infection day). The experimental period lasted from day 0 (infection) to day 28 post-infection (PI). On day 29 PI the lambs were humanely slaughtered as described below. The length of the PI experimental protocol was chosen to allow all infected animals to reach infection patency. The period of infection was not extended further because the main objective was to study worm establishment and because recent studies have suggested that Pelibuey lambs reared parasite free are able to start reducing their *H. contortus* fecal egg counts as early as day 26 PI (Méndez-Ortíz et al., 2022; Ojeda-Robertos et al., 2017; Ramos-Bruno et al., 2021a).

2.3.1. Feeding levels, intake and weight gain measurements

From day − 28 preinfection, the lambs were weighed and individually housed in metabolic cages with a raised floor. The animals were separated into three diet groups (n = 14) with different DWGs:

- Diet 1: DWG 75 g/d
- Diet 2: DWG 125 g/d
- Diet 3: DWG 200 g/d

The respective diets are shown in Table 1. Allocation was performed to balance the lamb groups by sex and BW. The lambs were weighed once weekly, and individual feed intake was monitored daily. To achieve the expected DWG of the individual lambs, the quantity of feed offered was adjusted weekly using a feed formulation program based on the AFRC (1993). The program considered the feed composition of each diet, the individual lamb's weight and the expected DWG of the respective diet. The feed quantity adjustment was performed in the same manner for infected and noninfected lambs.

2.3.2. Artificial infection

Ten lambs in each diet group were artificially infected with L_3 larvae

Table 1
Composition of experimental diets consumed by Pelibuey hair sheep lambs artificially infected with *Haemonchus contortus*.

	Diet 1 (DWG 75 g)	Diet 2 (DWG 125 g)	Diet 3 (DWG 200 g)
Ingredients (%)			
Maize grain meal	9.60	18.30	21.50
Soybean meal	5.40	10.70	19.90
Johnsongrass hay	65.60	49.00	35.90
Calcium carbonate	1.30	1.20	1.30
Molasses	14.80	17.10	18.50
Urea	0.90	0.90	0.00
Vegetable oil	2.40	2.70	2.80
Nutrient content			
Crude protein (%)	8.48	13.39	14.96
ME (MJ/Kg)	16.72	17	17
NDF (%)	63.38	48.32	45.2
Intake (% BW)	3.0	3.3	3.7

DWG: daily weight gain; ME: metabolizable energy; NDF: neutral detergent fiber; BW: body weight of *H. contortus* (450 L_3/kg BW, *per os*) (infected). The larvae were administered on two consecutive days (days 0 and 1 PI). The remaining four lambs in each group were kept as noninfected (NInf) controls throughout the study.

2.3.3. Parasitological analyses

To confirm that all animals remained free of GIN infection until artificial infection, individual fecal samples were collected and analyzed using the centrifugal flotation technique (Rodríguez-Vivas and Cob-Galera, 2005).

After artificial infection, feces were collected on days 7, 14, 21, 25 and 28 PI. These fecal samples were processed on the day of collection by centrifugal flotation and the McMaster technique (Bauer et al., 2010). Fecal samples were also obtained from the NInf animals on the same days and processed by the centrifugal flotation technique to confirm the absence of GIN eggs.

2.3.4. Blood analyses

Individual blood samples were collected from the jugular vein on days − 7, 0, 7, 12, 21 and 28 PI. Two blood samples were collected from each lamb: with and without anticoagulant. The serum from the sample without anticoagulant was separated and frozen (−20 °C) in 1.5-mL microvials until further processing.

The blood samples with anticoagulant (EDTA, Vacutainer®, Becton Dickinson, New York, USA) were used to estimate the number of peripheral eosinophils (EOS) and concentrations of hematocrit (Ht) and hemoglobin (Hb). The number of EOS was determined using Carpentier's technique (Dawkins et al., 1989); in brief, the blood was diluted 1:10 to hemolyze the sample, and the cells were stained with Carpentier's solution (1% eosin Y and 40% formaldehyde) and counted in a Neubauer chamber (Cruz-Tamayo et al., 2020; Cuenca-Verde et al., 2011). The EOS in 0.02 μL of blood were counted, and the results were multiplied by 50 to obtain the number of EOS per μL of blood as indicated by Voigt (2003). The capillary microhematocrit technique was used to determine Ht (Benjamin, 1991). The Hb concentration was assessed by spectrophotometry using the cyanomethemoglobin method (Exigo Vet®, Boul Medical AB, Spanga, Sweden).

2.3.5. Haemonchus contortus antigen preparation

Two hundred male adult *H. contortus* worms were macerated and pulverized in a mortar with liquid nitrogen to extract protein. The worms were subsequently homogenized in 10 mL of 0.05 M phosphate buffer (PBS), 1 mM PMSF, 50 mM DTT, and a cocktail of protease inhibitors (20 μL/mL; Roche, 04693132001). The homogenate was centrifuged at 4 ºC for 15 min (15,000 rpm), and the supernatant was stored at − 80 ºC until use.

The protein concentration of the sample was estimated by the Peterson method (Peterson, 1977). The DOC-TCA protein precipitation technique rapidly quantifies soluble and membrane proteins and eliminates interfering substances even in highly diluted solutions (<1 μg/mL protein). The total protein content was quantified by referring to a linear log-log protein standard curve constructed using bovine serum albumin.

2.3.6. Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was performed to determine the levels of *H. contortus*-specific IgG antibodies in serum from infected animals at different PI times. Serum from NInf animals was used as a control.

Polystyrene multiwell plates (Costar, cat. 3591) were coated with 100 μL of *H. contortus* antigenic extract (10 μg/mL) diluted in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed four times with Tween 20 (0.05% v/v) in PBS to remove unbound antigen. Nonspecific reactions were blocked with 200 μL of bovine serum albumin (3% w/v) in PBS for 1 h at 37 °C. The serum samples were diluted with PBS, and 100 μL of a 1:200 dilution was added to each well. The plates were incubated for 1 h at 37 °C. Three washes were performed with Tween 20 in PBS. The HRP-conjugated anti-sheep IgG antibody (2 mg/mL; Abcam, cat. Ab6747) was diluted with PBS, and 100 μL of a 1:3000 dilution was added to each well. The plates were incubated for 1 h at 37 °C. Three washes were performed with Tween 20 in PBS. The substrate (10 mL of citrate buffer (pH 5.0), 50 μL of hydrogen peroxide (2.5–3.5%), and 4–6 mg of *o*-phenylenediamine (OPD)) was incubated (100 μL) for 15 min at room temperature in the dark. The reaction was stopped by adding 35 μL of 2 M sulfuric acid to each well. The plates were read in a Multiscan FC ELISA plate reader (Thermo Scientific™, China) at a wavelength of 450 nm.

2.3.7. Humanitarian slaughter

On day 29 PI, all lambs were slaughtered according to Mexican national standards for the human slaughter of domestic animals (NOM-033-ZOO-2014). The midline of each animal was opened to ligate the abomasum and small and large intestines independently. The abomasum was removed from each animal and processed independently to obtain each organ's lumen contents and washings. These materials were sieved (sieve No. 200; MAFF, 1984) and preserved in 10% v/v formaldehyde (Gárate-Gallardo et al., 2015; Torres-Acosta et al., 2015).

2.3.8. Identification, counting and sexing of adult parasites

The worm counting process began by eliminating the formaldehyde from the collected abomasal contents and washings of each lamb using tap water and a sieve (No. 200). The sieved contents were deposited in a plastic container filled with 2 L of tap water. Next, a 10% subsample was collected in 5 aliquots of 40 mL (Gárate-Gallardo et al., 2015). Worm counting was performed in Petri dishes with equidistant parallel lines (1 cm) to facilitate observation under a stereomicroscope (12X). Adult male and female parasites were collected using dissecting needles. The total number of adult males or females recovered was multiplied by 10 to obtain the total number of parasites in each lamb (Gárate-Gallardo et al., 2015; Torres-Acosta et al., 2015). Confirmation of the parasitic species was performed on male parasites by observing their copulatory bursa, spicules and asymmetrical dorsal ray according to the identification keys by Barth and Visser (1991).

2.3.9. Length and fecundity of adult female parasites

Thirty *H. contortus* adult females with a gravid uterus were randomly selected from each animal, and their lengths were determined by observation under a stereomicroscope using a calibrated grid. Adult female fecundity was subsequently estimated by placing an individual female worm in a vial containing 0.5 mL of tap water. The specimen was macerated to release the eggs *in utero* (EIU), and the suspension was added to 0.5 mL of water and homogenized by vortexing for 10 s. The EIU counts of each worm were performed using five 20-μL aliquots under a compound microscope (40X objective). The total EIU number in the sample was multiplied by 10 to obtain the total number of eggs per female (Torres-Acosta, 1999).

2.4. Statistical analysis

The Shapiro-Wilk test confirmed the normality of the DWG and Ht data, and Levene's test was used to confirm their homogeneity of variance. The effects of diet, infection and their interaction on the mean DWG were compared by ANOVA using the GLM procedure. A *post hoc* comparison was performed using Tukey's test. Differences between means were considered significant when $P < 0.05$. The Ht values were subjected to same statistical procedure, but only a significant effect of infection was found. Thus, each dataset (infected and Ninf animals) was subjected to repeated-measures ANOVA to compare the effects of diet and infection time throughout the study. In each analysis, a *post hoc* comparison was performed using the Bonferroni test in Statgraphics Centurion XVI.

The data for Hb, EOS and IgG were not normally distributed, and hence the Box-Cox procedure was used to normalize these data (Statgraphics Centurion XVI). Once the normality of the data was verified, the effects of diet, infection and their interaction on the mean Hb, EOS and IgG for the whole study period were analyzed by ANOVA using the GLM procedure. A *post hoc* comparison was performed using Tukey's test. Differences between means were considered significant when $P < 0.05$. Repeated-measures ANOVA tests were subsequently performed to determine the effect of diet throughout the study on the Hb concentrations of the infected and the NInf lambs. The same procedure was performed for the EOS and IgG variables.

The effect of diet on EPG at days 25 and 28 PI, the length of female worms and the number of EIU were compared using the nonparametric Kruskal-Wallis test because the data were not normally distributed, even after the use of different transformation protocols. A *post hoc* comparison was performed using the median notches option in the box-and-whisker plot of Statgraphics Centurion XVI.

The data on the total adult worm burden (TAW), male and female adult worm burdens, and the ratio of adult females to males (F:M) did not meet the assumption of normality. Hence, the effect of diet on the worm burden parameters and the F:M ratio were compared pairwise (Diet 1 *vs* Diet 2, Diet 1 *vs* Diet 3 and Diet 2 *vs* Diet 3) using the nonparametric Wilcoxon signed-rank test.

Respective Spearman correlation coefficients were calculated between different parasitological data (EPG, TAW, female length and EIU) and the serum IgG data to determine the associations between these variables for each diet. Values of $P < 0.05$ were considered statistically significant.

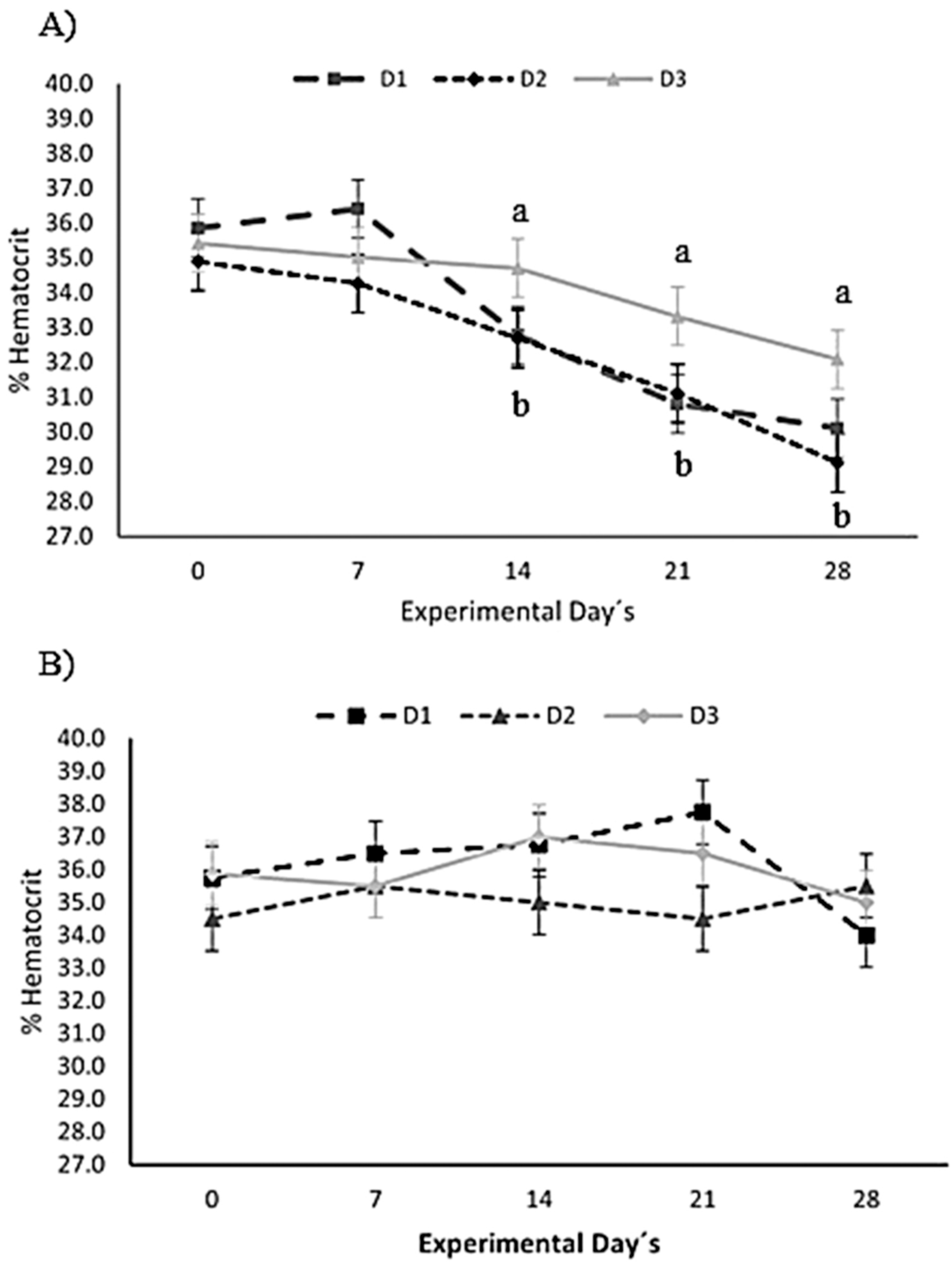

Fig. 1. Effect of three feeding levels (D1, D2, D3) on the dynamics of the mean hematocrit values (%) in *Haemonchus contortus*-infected (A) and noninfected (B) Pelibuey hair sheep lambs. The estimated weight gain for the experimental diets was D1: 75 g/day; D2: 125 g/day; and D3: 200 g/day. [ab] Different letters within each figure indicate significant differences ($P < 0.05$).

3. Results

3.1. Daily weight gain, hematocrit and hemoglobin values

As expected from the study design, the DWG of the lambs differed between the experimental diet groups ($P = 0.001$). However, there were no differences due to *H. contortus* infection or the diet × infection interaction. The animals consumed the experimental diets as planned, and no refusal was recorded. The DWG was 85 ± 3 g/d for Diet 1, 124 ± 4 g/d for Diet 2, and 210 ± 6 g/d for Diet 3.

The mean Ht values of infected lambs (33.2 ± 0.48%) during the study were lower ($P = 0.027$) than those of NInf lambs (35.7 ± 0.61%). However, there was also a significant effect of the diet × infection interaction. From day 14 onward, lambs consuming Diets 1 or 2 had lower Ht levels than lambs consuming Diet 3 (Fig. 1A; $P = 0.044$). By contrast, the NInf lambs' Ht values were similar among the three experimental diets throughout the study (Fig. 1B).

For all three experimental diets, Hb values were lower in infected lambs than in NInf lambs ($P = 0.004$). By contrast, no effect of diet or the diet × infection interaction on Hb values was observed. The dynamics of mean Hb values showed a significant infection × time interaction ($P = 0.001$) indicating lower Hb levels on days 21 and 28 PI (Fig. 2), irrespective of the experimental diet. The NInf lambs maintained constant Hb levels throughout the study regardless of diet.

3.2. Egg count in feces

Table 2 shows the median EPG counts of the infected lambs on each experimental diet on days 21, 25 and 28 PI. No statistical comparison of the EPG count on day 21 PI between the diets was performed because the infected lambs were at the prepatent stage of infection on day 21 PI, as confirmed by flotation analyses. The EPG values on days 25 and 28 PI showed that lambs on Diet 3 had lower EPG than lambs consuming Diets 1 or 2 ($P = 0.002$). By contrast, the NInf lambs remained free of *H. contortus* infection throughout the experiment.

3.3. Adult H. contortus burdens

The total adult *H. contortus* burden (TAW) and total male and female burdens are shown in Table 3. Lambs consuming Diet 3 had fewer TAW and male parasites than lambs consuming Diets 1 or 2 (Diet 1 *vs* 3 $P = 0.037$; Diet 2 *vs* 3 $P = 0.049$), and lambs consuming Diet 3 had fewer adult female worms than lambs consuming Diet 2 ($P = 0.037$). However, the *H. contortus* female to male ratio (F:M) was similar among the lambs irrespective of the experimental diet (Table 3).

The TAW count was used to estimate the percentage of L_3 that reached the adult stage. The median percentage of L_3 larvae reaching the adult stage was 13.8% (min: 0.13% and max: 42.13%) in lambs consuming Diet 1, 7.8% (min: 3.87% and max: 58.26) in lambs consuming Diet 2 ($P = 0.970$), and 4.0% (min: 0.01% and max: 13.63%) in lambs consuming Diet 3 ($P = 0.037$).

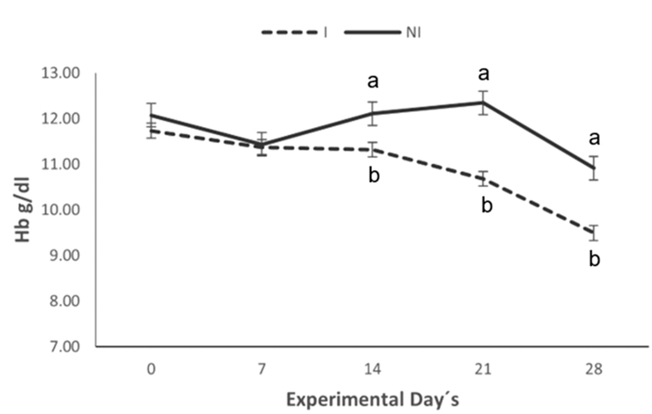

Fig. 2. Effect of *Haemonchus contortus* infection on the dynamics of the mean hemoglobin values (Hb g/dL) in Pelibuey hair sheep lambs. [ab] Different letters at each sampling point in each figure indicate a significant difference ($P < 0.05$).

3.4. Peripheral eosinophil counts and IgG values

The EOS values were higher in the infected lambs than in the NInf lambs ($P = 0.001$), regardless of diet or the diet × infection interaction. The mean EOS values of the infected lambs (Fig. 3) increased from day 7 PI and remained high until the end of the study irrespective of the diet (day 28 PI). By contrast, the mean EOS count remained < 200 cells/mL in Ninf lambs (Fig. 3).

The mean values of serum IgG against *H. contortus* were higher in the infected lambs than in the NInf lambs ($P = 0.001$). These values were not affected by diet or the diet × infection interaction ($P = 0.001$). Fig. 4 shows the increase in IgG against *H. contortus* in the infected lambs from day 14 PI until the end of the study. By contrast, all the NInf lambs remained negative (IgG values < 400 nm OD) throughout the study, and no significant difference in IgG values was observed between diet groups.

A negative correlation was detected between IgG and EPG values on days 0, (−0.685, $P = 0.039$) 21 PI (−0.818, $P = 0.014$) and 28 PI (−0.685, $P = 0.039$) in lambs consuming Diet 1. Meanwhile, lambs consuming Diet 3 showed a negative correlation between IgG and EPG on day 21 PI (−0.846, $P = 0.011$) and between IgG on day 21 PI and TAW (−0.723, $P = 0.030$). By contrast, no correlation of serum IgG levels with parasite length or EIU was found (Table 4).

3.5. Length of H. contortus females and eggs in utero

The length of *H. contortus* females collected from experimental lambs was not affected by diet ($P > 0.05$). The median length of adult females was 1.8 cm (min: 1.2 cm, max: 2.35 cm) among the three diet groups. The EIU of adult female worms was similar for lambs consuming Diets 1 (median: 182 EIU, min: 5 and max: 865) and 2 (median: 197 EIU, min: 30 and max: 880). By comparison, female worms obtained from lambs consuming Diet 3 had fewer EIU (median: 150 EIU, min: 10 and max: 640) ($P = 0.004$).

4. Discussion

Previous studies of protein supplementation in lambs shaped a paradigm that has remained unchanged since the early 1990 s and cited in numerous reviews: dietary manipulation cannot modulate the establishment of *H. contortus* in 'parasite naïve' lambs in their first infection (Holmes, 1993; Coop and Holmes, 1996; Coop and Kyriazakis, 1999; Hoste et al., 2016). However, new evidence has emerged in recent decades that the immune responses of lambs differ between hair sheep breeds and other sheep breeds (González et al., 2008, 2011, Hernández et al., 2016). The present trial is the first to study the effect of feeding level on the establishment of *H. contortus* using parasite-naïve Pelibuey hair sheep lambs, a short-haired breed reared for meat.

4.1. Impact of diet and infection on weight gain and blood variables

This study confirmed that Pelibuey hair sheep lambs are resilient to high levels of adult *H. contortus* during their first infection as reported in previous studies (Galicia-Aguilar et al., 2012; Méndez-Ortíz et al., 2012; Méndez-Ortíz et al., 2022). *H. contortus* infection did not result in any clinical signs of haemonchosis, such as anorexia, submandibular or ventral edema, anemia, weakness or scant dark feces (Besier et al., 2016). In addition, the infection did not reduce the expected DWG of lambs irrespective of diet group, confirming that the cost of subclinical exposure to *H. contortus* was low relative to the body's nutrient

Table 2
Effect of three feeding levels on the excretion of *Haemonchus contortus* eggs per gram of feces in artificially infected Pelibuey hair sheep lambs.

		21 DPI*		25 DPI		28 DPI	
Diets	N	Median	Range	Median	Range	Median	Range
1 (75 g/day)	10	2850	0–8700	7900[a]	50–14000	7000[a]	100–15650
2 (125 g/day)	10	1100	0–8750	3700[a]	750–20650	3400[a]	300–25450
3 (200 g/day)	10	550	0–5350	925[b]	0–3850	625[b]	0 – 1650

[ab] Different letters in the same column indicate a significant difference ($P < 0.05$)
DPI: days post-infection
* Day 21 post-infection was not compared statistically

Table 3
Effect of three feeding levels on *postmortem* counts of *Haemonchus contortus* adults collected from artificially infected Pelibuey hair sheep lambs.

		Total adult parasites		Total male parasites		Total female parasites		Female:Male Ratio	
Diets	N	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
1 (75 g/day)	10	1717.5[a]	21 – 5593	876.5[a]	10 – 2706	892.0[ab]	11 – 2887	1.08[a]	0.822–1.434
2 (125 g/day)	10	854.5[a]	463 – 8652	390.5[a]	223–4338	488.5[a]	192 – 4314	1.08[a]	0.708 – 2.152
3 (200 g/day)	10	472.5[b]	2 – 1736	216.5[b]	1–710	283.0[b]	1–1026	1.30[a]	0.938 – 2.905

[ab] Different letters in the same column indicate a significant difference ($P < 0.05$).

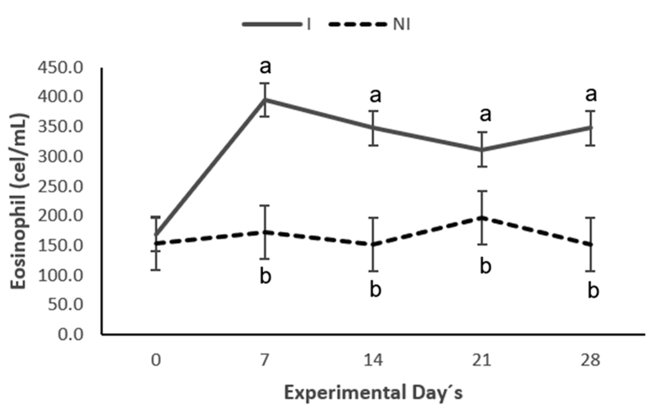

Fig. 3. Effect of *Haemonchus contortus* infection on the dynamics of the mean peripheral eosinophil values (eosinophils, Cell/mL) in Pelibuey hair sheep lambs. [ab] Different letters at each sampling point in each figure indicate a significant difference ($P < 0.05$).

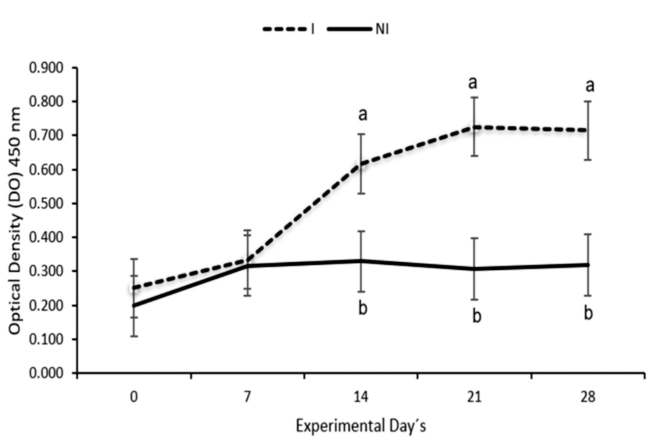

Fig. 4. Effect of *Haemonchus contortus* infection on the dynamics of the mean IgG values (optical density, OD) in Pelibuey hair sheep lambs. [ab] Different letters at each sampling point in each figure indicate a significant difference ($P < 0.05$).

Table 4
Spearman correlations between IgG optical density values and parasitological variables in Pelibuey hair lambs artificially infected with *Haemonchus contortus*.

DPI	N	EPG	TAW	Length	EIU
Diet 1 (75 g/day)					
0	10	-0.685 *	-0.648	0.418	0.248
7	10	-0.642	-0.636	0.272	0.272
14	10	-0.642	-0.563	0.248	0.260
21	10	-0.818 *	-0.648	0.515	0.224
28	10	-0.685 *	-0.442	0.317	0.785
Diet 2 (125 g/day)					
0	10	0.066	0.512	-0.381	-0.564
7	10	0.091	0.382	-0.309	-0.442
14	10	-0.079	0.333	0.030	-0.285
21	10	0.103	0.527	-0.018	-0.467
28	10	0.018	0.393	0.224	-0.491
Diet 3 (200 g/day)					
0	10	-0.006	-0.018	0.103	-0.328
7	10	-0.580	-0.541	0.553	-0.024
14	10	-0.469	-0.274	0.249	0.170
21	10	-0.846 *	-0.723 *	0.601	0.377
28	10	-0.451	-0.492	0.371	0.258

DPI: days post-infection; EPG: eggs of *H. contortus* per gram of feces; TAW: total adult parasites; Length: female parasite length; EIU: eggs of *H. contortus in utero*.
* P value: Diet 1 (DPI 0, EPG = 0.039; DPI 21, EPG = 0.014, DPI 28, EPG = 0.039), Diet 3 (DPI 21, EPG = 0.011, DPI 21, TAW = 0.030).

requirements for energy and protein. In other words, the nutritional cost of GIN infection was not reflected in DWG (Méndez-Ortíz et al., 2019; Ramos-Bruno et al., 2021a, b). Hence, the recorded differences in DWG between the diet groups were consistent with the expected differences in performance based on the procedures recommended by the AFRC (1993). The same feed formulation methodology was previously used for other pen studies of tropical hair sheep lambs (Méndez-Ortíz et al., 2019; Ramos-Bruno et al., 2021a, b).

Because *H. contortus* are hematophagous, the impact of infection on Ht and Hb values was evaluated (Besier et al., 2016). Regardless of diet and infection status, Ht remained above the anemia threshold in all lambs during the entire experiment (> 20% of Ht; Kaplan et al., 2004). The maintenance of Ht values is a sign of resilience to *H. contortus* infection and has been reported in several artificial infections pen studies with parasite-naïve hair sheep (Galicia-Aguilar et al., 2012; Méndez-Ortíz et al., 2022; Ramos-Bruno et al., 2021a). However, in infected lambs, Ht decreased from day 14 PI onward, regardless of diet. The reduction in mean Ht in infected lambs was larger in lambs consuming Diets 1 or 2 than in lambs consuming Diet 3 (Fig. 1A). The smaller decrease in the mean Ht in the lambs consuming Diet 3 was supported by the significant diet × time interaction and can be explained by the fact that Diet 3 provided the largest quantity of nutrients compared with the other diets. The lambs consuming Diet 3 seemed able to use the extra nutrients to compensate for the blood loss caused by *H. contortus* infection (Hoste et al., 2016). In addition, the lambs fed Diet 3 had significantly lower worm burdens, as will be discussed below, which could have resulted in milder mucosal damage and less blood loss compared with the lambs consuming the other experimental diets.

Regardless of diet and infection status, Hb remained above the anemia threshold in all lambs during the entire experiment (> 9 g/dL; Byers and Kramer, 2010). The Hb values of the lambs were only affected by infection and the infection × time interaction. No significant effect of diet or the diet × time interaction was found. The reduction in Hb in infected lambs compared with Ninf lambs became more pronounced as the study progressed, irrespective of diet.

In summary, the infected lambs exhibited declines in Ht and Hb as the study progressed (after day 14 PI), but the animals showed resilience, as these effects of infection never manifested as overt clinical signs or reduced DWG.

4.2. Impact of diet and infection on peripheral eosinophils and serum IgG values

EOS increased significantly in infected lambs compared with NInf lambs as early as day 7 PI, and this effect was maintained until the end of the study. This increase suggests a rapid innate immune response of the lambs to *H. contortus* infection. Increased EOS in infected lambs was reported previously for hair sheep (Cruz-Tamayo et al., 2020) and differentiates the immune response of Canarian hair sheep from that of Canarian wool-type sheep (González et al., 2011; Hernández et al., 2016). However, the increase in EOS was similar in all infected lambs, regardless of the experimental diet. Therefore, EOS alone cannot explain why infected lambs on Diet 3 had fewer adult parasites or EIU than infected lambs consuming poorer diets. In this context, it is important to estimate the number of eosinophils in the abomasal mucosa, which, together with abomasal mast cells, could help explain the effect of diet on the number of parasites reaching the adult stage (Lacroux et al., 2006).

No increase in IgG levels was detected in the NInf lambs, consistent with their persistent worm-free status throughout the entire experiment. The significant increase in IgG levels in the infected lambs was independent of the experimental diet, suggesting that all mounted a humoral response mediated by IgG isotype antibodies that started after day 7 PI and remained stable until day 28 PI. These results are consistent with previous studies of Gulf Coast native sheep, which are also considered *H. contortus* resistant (Shakya et al., 2011). An inverse correlation was found between IgG and EPG in lambs consuming Diet 1 on days 21 and 28 PI. For lambs on Diet 3, negative correlations of IgG with EPG (day 21 PI) and TAW were found. These results suggest a biological influence of IgG antibodies on parasitological variables (EPG, TAW), as documented by Gómez-Muñoz et al. (1999), who reported that an increased IgG level is associated with decreased EPG and adult parasite burdens. Other studies in sheep have detected increased levels of IgE and IgA in the response of sheep to GIN infection (Pfeffer et al., 1996; Kooyman et al., 1997; Shaw et al., 1998; Salgado et al., 2022). It has even been suggested that IgA can be used to classify animals as resistant or susceptible to these parasites (Salgado et al., 2022). IgE and IgA warrant further investigation.

4.3. Impact of feeding level on fecal egg excretion and postmortem parasite counts

This study is the first to demonstrate that Pelibuey hair sheep lambs raised GIN-free can reduce the *H. contortus* worm burden when consuming a diet allowing DWG > 200 g/d (Diet 3). These lambs were able to reduce their worm burden by 48.7% compared with lambs consuming Diet 2 and by 71% compared with lambs consuming Diet 1. Previous artificial infection studies of Scottish Blackface lambs, Finn Dorset lambs and Hampshire lambs did not find a decrease in the establishment of *H. contortus* as dietary protein increased (Abbott et al., 1985; Wallace et al., 1995; 1996). As noted by González et al. (2008), some indigenous hair sheep breeds possess natural resistance to GIN and may represent a valuable genetic resource that can be used to study the mechanisms of protection against GIN. Hair sheep breeds with natural resistance to GIN infections include Santa Ines, Barbados Blackbelly, Saint Croix, Florida Native, Pelibuey and Red Maasai (Amarante et al., 2009; Jacobs et al., 2016; Zvinorova et al., 2016; Estrada-Reyes et al., 2017). These indigenous hair sheep breeds have been naturally selected for hundreds of years to survive in the hot, humid tropical environment where *H. contortus* infections are common. The Caribbean hair sheep breeds show a stronger inflammatory response against *H. contortus*, which can explain the increased resistance of these breeds to this parasite (MacKinnon et al., 2009).

The lower worm burdens recorded for lambs on Diet 3 were even evident *antemortem*, as they had lower EPG counts than lambs consuming Diets 1 or 2 (Table 2). This relationship was expected because of the strong positive correlation between EPG and adult female *H. contortus* numbers (> 70.4%) reported previously for artificially infected Pelibuey sheep (Ramos-Bruno et al., 2021a). In addition to the reduction in worm burden, the female parasites obtained from lambs consuming Diet 3 had significantly fewer EIU than female parasites from lambs consuming Diets 1 or 2. The reduction in EIU also helps explain the lower EPG excretion by lambs consuming Diet 3, which has been attributed to abomasal eosinophils (Terefe et al., 2005). By contrast, the experimental diets did not influence the length of the female adult parasites. In general, the length of adult female *H. contortus* is inversely related to the number of adult parasites present in the abomasum (Gárate-Gallardo et al., 2015). However, in the present study, no association was found between the size and population density of worms.

The present study demonstrated that a diet designed to allow DWG > 200 g/d reduced *H. contortus* worm burdens and the pathophysiological impact of infection in Pelibuey hair sheep lambs. These findings open the possibility of using nutritional manipulation as an alternative method to modulate *H. contortus* L_3 establishment in Pelibuey lambs. Farmers using this breed could maintain their weaned lambs at a high growth rate (> 200 g/d) when first exposed to grazing or browsing, particularly during the rainy season, when infectivity is often highest (Jaimez-Rodríguez et al., 2019). Such a strategy could help limit the number of established parasites in lambs, which implies a lower cost of infection, while also improving the lambs' premunity against GIN. However, growth rates > 150 g/d are very difficult to achieve for Pelibuey lambs under field conditions unless a large proportion of grain supplement is used (Retama-Flores et al., 2012). A supplementation strategy should only be implemented after a careful cost-benefit analysis that considers the cost of feed *versus* the benefits of resilience, resistance, and the avoidance of anthelmintic resistance, which should be confirmed on a farm-by-farm basis. Thus, before implementing a supplementation strategy, farmers and their advisers must identify a supplement source that is readily available at a suitable price. In addition, the feed must be offered constantly to maintain high growth rates after weaning. The latter is a difficult task for most farmers.

Further studies are required to confirm whether the findings for Pelibuey hair sheep lambs are applicable to other hair sheep breeds, which could be advantageous for raising hair sheep breeds under hot tropical and subtropical conditions. Confirmation might help revalorize the indigenous GIN-resistant breeds, which are largely neglected in their own region of origin and readily exchanged for exotic breeds.

5. Conclusion

All of the diets examined in the present study enabled resilience of Pelibuey lambs to *H. contortus* infection. However, the diet designed for a DWG of 200 g/d reduced the adult worm burdens in Pelibuey lambs. This diet also resulted in improved Ht and reduced the EPG, TAW and EIU of female worms. Our findings strengthen the importance of adequate nutrition, both in quality and quantity, for coping with *H. contortus* infections.

Ethics statement

The Bioethics Committee of the Campus of Biology and Agricultural Sciences - Autonomous University of Yucatan, presided by Antonio Ortega Pacheco, PhD, approved the present study with notification letter CB-CCBA-I-2020–001.

CRediT authorship contribution statement

A. Can-Celis: Conceptualization, Data collection, Data curation, Formal analysis, Investigation, Writing – original draft. **F.A. Mendez-Ortiz:** Conceptualization, Data collection, Data curation, Formal analysis, Investigation, Writing – original draft; Writing – review and editing, Supervision. **J.F.J. Torres-Acosta:** Conceptualization, Data collection, Data curation, Formal analysis, Investigation, Writing – original draft; Writing – review and editing, Supervision. **M.G. Mancilla-Montelongo:** Data collection, Data curation, Investigation. **P.G. Gonzalez-Pech:** Data collection, Data curation, Investigation. **E. Ramos-Bruno:** Data collection, Data curation, Investigation. **C.A. Sandoval Castro:** Formal analysis, Investigation, Writing – review and editing. **J.J. Vargas-Magaña:** Data collection, Data curation, Investigation, Writing – review and editing. **F. Bojórquez-Encalada:** Data collection, Data curation. **A. Cruz-Tamayo**: Data collection, Data curation, Investigation. **E. Canché -Pool:** Data collection, Data curation, Investigation, Writing – review and editing. **M.E. López-Arellano:** Investigation and methodology. **R.M.Galaz-Ávalos:** Investigation and methodology. **V. Loyola-Vargas:** Investigation and methodology.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Data availability

None of the data have been deposited in an official repository. Data will be made available upon reasonable request.

Acknowledgments

F.A. Méndez-Ortíz thanks the Program for Professional Teacher Development (PRODEP-SEP, Mexico) and the Autonomous University of Campeche for their support for project UNACAM-EXB-113. We thank Dr. Jorge González and his colleagues at the University of Las Palmas de Gran Canaria for advice on immunological techniques.

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