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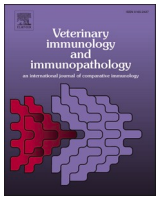

Haemonchus contortus parasitic stages development and host immune responses in lambs of different sheep breeds

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ABSTRACT

Variable host resistance against *Haemonchus contortus* infection was extensively associated with sheep breed, but the mechanisms responsible for the enhanced resistance remains scarcely elucidated. The aim of the present study was compare the phenotypic profile of three breeds (Santa Inês, Texel and White Dorper) with the relative expression of immune-related genes in the abomasal mucosa of sheep breeds infected with *H. contortus*. Besides, these phenotypic profiles were compared with relative expression of immune related genes in the animal abomasum. Decreasing resistance against *H. contortus* infection among sheep breeds was observed in the following order: Santa Inês, Texel and White Dorper. Differential local immune responses were developed during chronic infection, wherein both Santa Inês and White Dorper presented high activity of innate receptors, especially *TLR2*, while Th2 related transcripts trended to be superior in the Texel lambs. The White Dorper lambs also presented increased local inflammation since most inflammatory related genes, including the pro-inflammatory mediators *NFKBIA* and *IL1B*, and anti-inflammatory cytokines *IL10* and *TGF* were upregulated. The host responses to different parasite stages were characterised by *TLR2* activity during earlier stages, while complement activity (*CFI*) was involved in the clearance of latter parasite stages. Further, *TLR4* activity affected the responses to both early and late parasite stages. To our knowledge, this is the first study to point out for differential immune responses among sheep breeds and to different *H. contortus* parasitic stages. The better elucidation of these host-parasite interactions may improve the immune-prophylactic management of haemonchosis.

1. Introduction

Haemonchus contortus is the most pathogenic parasite that affects sheep, especially in tropical and subtropical climates. Multiple anthelmintic resistance to this gastrointestinal nematode (GIN) is widely disseminated and rapidly increasing (Arsenopoulos et al., 2021; Bassetto et al., 2024; Kotze and Prichard, 2016). Therefore, alternative measures for GIN control are highly required to reduce sheep production losses, including selective breeding for parasite resistance and development of immune-prophylactic management (Amarante et al., 2004; Louvandini et al., 2006).

Regarding the natural *H. contortus* resistance associated with sheep breed, hair type animals, as Santa Inês, Morada Nova and Red Maasai breeds, present well known superior resistance and/or resilience against this nematode infection, compared to wool types, as Ile de France and Sulfolk, or semi-wool type breed, as Dorper (Amarante et al., 2004; Chagas et al., 2024; Kapritchkoff et al., 2024; Mugambi et al., 1997; Toscano et al., 2019; Wanyangu et al., 1997). In a comparative study regarding haemonchosis resistance in ewes of different sheep breeds, resistance levels were decreasing from Santa Inês, Texel to Ile de France

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and to White Dorper (Gonçalves et al., 2018). In a recent study, Texel and Dorper ewes presented similar resistance, which was inferior to Santa Inês, while in lambs, Santa Inês presented the highest resistance, followed by Texel, and White Dorper was the most susceptible group (Kapritchkoff et al., 2024).

Besides the inter-breed variation associated with parasite resistance, some intra-breed features were also pointed out to lead to altered resistance against infection. Divergent *H. contortus* resistance phenotypes in Morada Nova lambs were associated with differential cell mediated local immune responses, wherein resistant animals presented increased responses mediated by specific antibodies, pattern of recognition receptors (PRRs), alarmins and complement, while susceptible animals developed an increased local inflammation (Toscano et al., 2019; Barbosa Toscano et al., 2020). Further, polymorphisms in the β-globin gene, which encodes the hemoglobin molecule, were associated with resistance against haemonchosis, and recently, with increased Th2 response, mucin dynamics and lectin activity (Kapritchkoff et al., 2024; Okino et al., 2021a, 2021b, 2023).

Sex differences also affected the development of humoral responses against haemonchosis, and consequently the resistance level to infection. Morada Nova female lambs, which were highly resistant against *H. contortus* infection, presented robust humoral responses mediated by specific IgM immunoglobulins, while in males, the IgG isotype was the most abundant. In this same study, PCV and FEC levels were significantly correlated (positive and negative, respectively) with the specific IgM serum titters (Okino et al., 2024).

In brief, the life cycle of *H. contortus* includes six stages: egg, four larval stages and adult. Each adult female can lay from 1000 to 5000 eggs per day, which are eliminated through faeces to pastures. The eggs hatch to free-living L_1 larvae stage, followed by L_2 and infective L_3 stage within 1–7 days. The L_3 in the pasture are ingested by the sheep, exsheathed in the rumen, migrate to the abomasum and moulted to the parasitic L_4 stage after 2–3 weeks. The L_5 stage, fixed to the abomasal mucosa, achieves the final adult parasite stage (Naeem et al., 2021).

The present study aimed to compare the phenotypic profile of three breeds (Santa Inês, Texel and White Dorper) with the relative expression of immune-related genes in the abomasal mucosa of sheep breeds infected with *H. contortus*.

2. Material and methods

2.1. Experimental design

Twenty-three male lambs (8 Santa Inês, 7 Texel, and 8 White Dorper) from the Embrapa Pecuária Sudeste experimental farm were born in two separate matings: 2022 (n = 12) and 2023 (n = 11). The lambs were raised in an endemic area for *H. contortus* and monitored for faecal egg counts (FEC) and packed cell volume (PCV) by microhematocrit method, at approximately 63, 84 (weaning day), 105, 126, 147, 168 and 189 days of age. All procedures have been approved by the Embrapa Pecuária Sudeste Ethical Committee for Animal Experimentation (process n. 02/2022), in accordance with ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation. Approximately at 210 days of age, blood samples from all animals were collected from jugular vein using vacutainer tubes containing EDTA and submitted to determination of PCV and complete hemogram, faeces were evaluated for FEC, and the lambs were subjected to euthanasia and necropsy. Samples of the fundic region of abomasum were collected, snap frozen in liquid nitrogen and stored at −80°C until processed. Ten percent of abomasal content and mucosa were submitted to worm classification counting (Burden et al., 2024). The *H. contortus* stages were morphologically differentiated and quantified into: early L_4 larvae, female L_4 larvae, male L_4 larvae, male L_5 larvae, female L_5 larvae, male adult worm and female adult worm (Ueno and Gonçalves, 1998).

2.2. RNA extraction and RT-qPCR for gene expression quantification

Total RNA was extracted from abomasal samples using QIAzol® Lysis Reagent (Qiagen) and Tissue Ruptor (Qiagen), followed by RNA purification in silica columns using RNeasy Mini Kit (Qiagen). The concentration and purity of the RNA samples were estimated by spectrophotometry (NanoDrop™ 2000, Thermo Scientific), and RNA integrity was confirmed by 1.5 % agarose gel electrophoresis through visual assessment of 28S and 18S ribosomal RNA. RNA (1800 ng) samples were treated with RQ1 RNAse-Free DNAse (Cat. M6101, Promega) and subjected to cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, cat. 4368814) and oligo(dT) primers in a T100™ Thermal Cycler (Bio-Rad). All procedures were carried out following the manufacturer's recommendations.

Real time quantitative PCR (qPCR) was performed using qPCRBIO SyGreen Mix (PCR Biosystems) and QuantStudio 6 Pro (Thermofisher) (FAPESP shared research equipment 2021/11830–0). The reaction mixture contained 20 ng of transcribed RNA, 5 μL of 2X qPCRBIO SyGreen Mix, 100 nM of ROX reference dye, 0.3 μM of forward and reverse primers in a final volume of 10 μL. The cycling conditions were: pre-incubation at 95 ºC for 2 minutes and 40 cycles of 95 ºC for 15 seconds and 60 ºC for 10 seconds, followed by melting curve analysis, with temperatures ranging from 60 to 95 ºC at 0.1 ºC per second in the continuous method. All samples were tested in duplicate, no-template controls (NTC) and RT *minus* sample were included in each qPCR run. The threshold values were manually set to a fixed value for each gene assay. The sequence of primers and efficiency of qPCR reactions were previously determined (Toscano et al., 2019; Barbosa Toscano et al., 2020; Okino et al., 2023; Toscano et al., 2018) (Supplementary information 1). Gene expression results for each target gene were normalized by the geometric mean of *GAPDH, B2M, PPIA* and *YWHAZ*, since among six reference candidate genes (*GAPDH*, *PPIA, YWHAZ*, *G6PDH*, *B2M* and *ACTB*) tested in this experiment, *GAPDH* and *YWHAZ* were the most stable in NormFinder ranking, *B2M* and *PPIA* were the most stable in the Genorm ranking, while *GAPDH* and *PPIA* were the most stable in Bestkeeper software. The relative gene expression was determined as described by Livak and Schmittgen (2001). For each gene combination, the sample with the lowest expression level (highest ΔCq) was adopted as a calibrator. Twenty target genes related to different pathways on response against *H. contortus* were tested: alarmins (*IL33*), pro-inflammatory cytokines (*IL1B* and *TNFA*), signaling of inflammatory response (*NFKBIA*), anti-inflammatory response (*TGFB* and *IL10*), Th2 profile cytokines (*IL5*, *IL13*, and *IL4*), complement molecules (*CFI* and *C7*), toll-like receptor 2 (*TLR2, TLR4,* and *TLR7*), lectin receptors (*GAL11* and *GAL14*), IgE receptor (*MS4A2*) and genes related to mucin activity (*TFF3*, *CLCA1*, and *B3GNT3*). Since some samples presented no amplification, the Cq values were set as 40 (total number of qPCR cycles) to calculate the relative expression, or for genes presenting few positive samples (*IL1B* and *IL13*), a descriptive/qualitative evaluation was performed.

2.3. Statistical analysis

Analyses were conducted in R software (R Core Team version 4.4.0, 2024). Fold change values of *GAL14, TGF, B3GNT3, TLR7, TLR4,* and *C7* genes were normalized with the log_x(.) transformation, while the worm counts and fold change values of *IL4* data underwent a boxcox(.) transformation (bestNormalize package; Peterson, 2021). Cubic root transformation normalized FEC data. All the transformed data were represented in graphs with "t" before the parameter name. The FEC and PCV results obtained from 63 to 189 days of age were evaluated using repeated measures ANOVA, with animals as the within-subjects variable and breed, year and collection date (interval) as between-subjects variables. The results of PCV, FEC, parasite counts and gene expression were also evaluated by ANOVA, with animals as the within-subjects variable and breed and year as between-subjects variables. The

ANOVA was performed using the anova_test(.) function of the rstatix package (Kassambara, 2023). Some outlier samples were removed from the analysis as described in Supplementary material 2. Duncan's multiple comparison test and residual analysis were performed using the ea2(.) function with design = 7 from the easyanova package (Arnhold, 2013). Correlation coefficients (ρ) were estimated by Pearson test between phenotypic values and gene expression levels (obtained in the euthanize date) (metan package; (Olivoto and Lúcio, 2020)). All the analyses were conducted using RStudio, and the significance level was set as $p \leq 0.05$. Due to the exploratory nature of present study, aiming to identify potential trends rather than confirm definitive results, a less stringent level ($p \leq 0.1$) of ANOVA analysis were also included in this study as potential trends.

3. Results

3.1. Phenotypic parameters from 63 to 189 days of age

Significant interactions were observed between lamb age and year ($p = 0.0438$) for FEC values. FEC During the second experimental year, FEC values were significantly higher at 63, 84 and 168 days of age (p-values = 0.011, 0.0413 and 0.0108, respectively) compared to the same ages of the first year (Fig. 1a). The White Dorper lambs presented the highest FEC values compared to other sheep breeds ($p < 0.01$) (Fig. 1b).

There were no significant interactions among age, year and breed for packed cell volume (PCV) values. The lowest PCV means were observed at 63, 84, 168 and 189 days of age (Fig. 1c). PCV levels were lower in the second experimental year compared to the first one ($p < 0.001$) (Fig. 1e). All the sheep breeds presented significantly different PCV levels ($p < 0.01$), wherein Santa Inês presented the highest means, followed by Texel and White Dorper (Fig. 1d).

3.2. Phenotypic parameters on euthanasia day

There were no significant differences in FEC values among sheep breeds (Supplementary information 3), while PCV was significantly superior for Santa Inês compared to Texel breed ($p = 0.0195$), and White Dorper lambs presented similar and intermediated values compared to both Santa Inês and Texel (Fig. 2a). Regarding the complete hemogram, hematocrit and hemoglobin values were significantly superior in Santa Inês compared to both Texel (p-values = 0.0036 and 0.0023, respectively) and White Dorper (p-values = 0.0006 and 0.0047, respectively) breeds (Figs. 2b and 2c), while no breed differences were detected for total leukocytes, neutrophils, eosinophils, lymphocytes and platelets (Supplementary information 3). Since monocyte and erythrocyte counts were affected by the collection year, these parameters were excluded from the analysis.

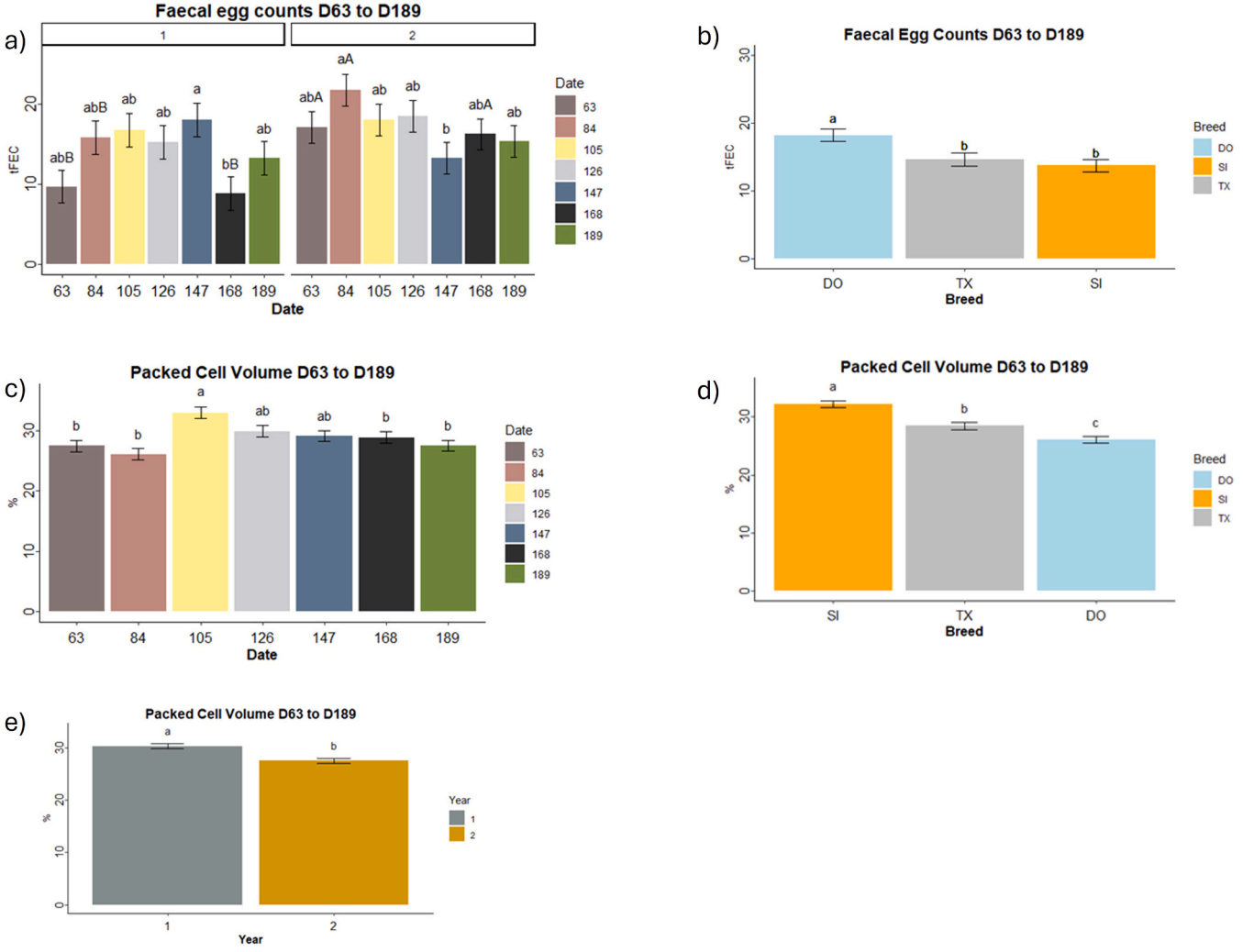

Fig. 1. Mean values of faecal egg counts and packed cell volume from 63 to 189 days of age, compared among lambs age (a and c), breed (b and d) and experimental year (e).

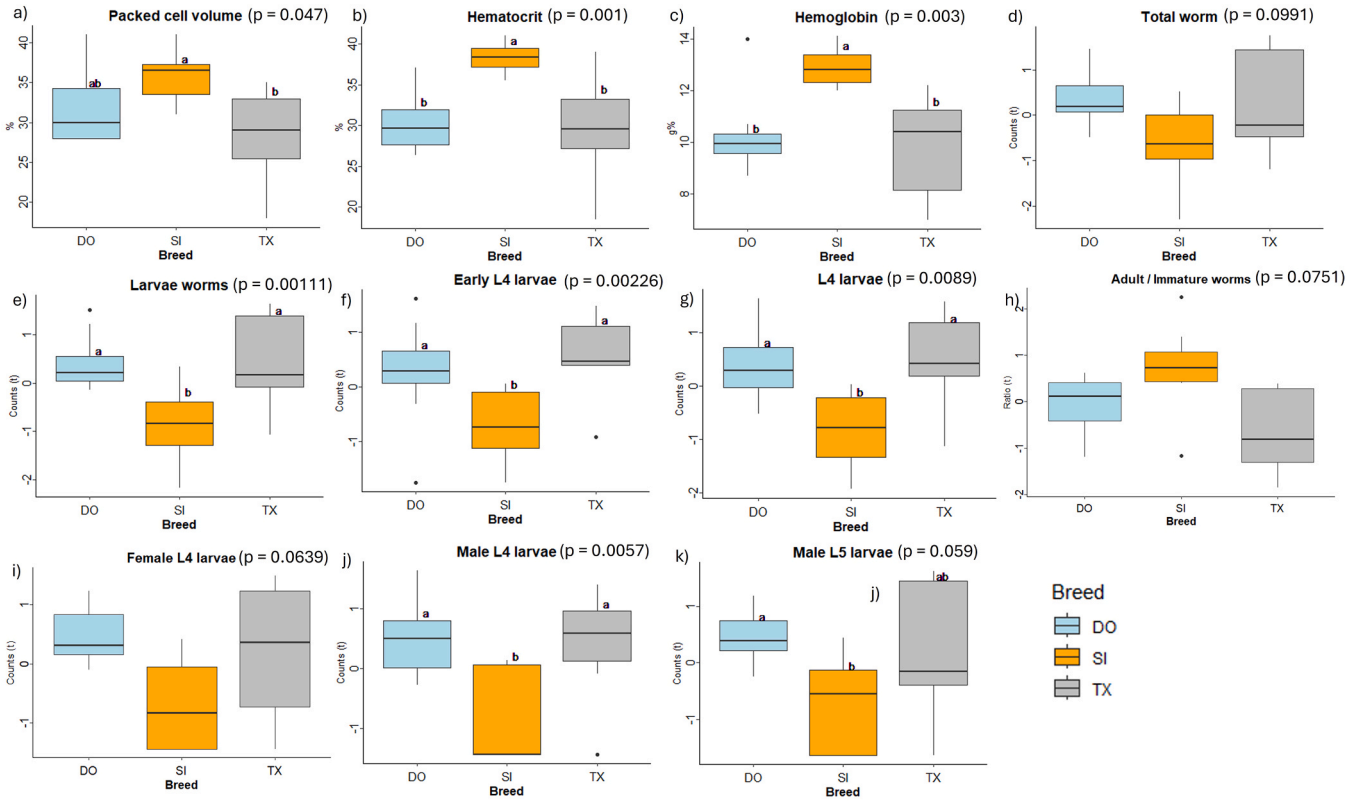

Fig. 2. Packed cell volume (PCV) by microhematocrit method (a); levels of hematocrit (b) and hemoglobin (c) by complete hemogram analysis; and parasitic stage counts in the abomasum, including: total (d), larvae (e), early L_4 larvae (f), L_4 larvae (g), adult/immature worm ratio (h), female L_4 (i), male L_4 (j) and male L_5 (k) of White Dorper (DO – blue bars), Santa Inês (SI – orange bars) and Texel (TX – grey bars) lambs of approximately 210 days of age naturally infected with *Haemonchus contortus*. The presented p-values are derived from the ANOVA test. Different letters among the sheep breeds indicated significant differences by Duncan test ($p < 0.05$). The "t" letter in the y-axis title indicated transformed data.

3.3. Worm counts in abomasum

There were no significant differences in the specific worm counts of adult and L_5 larvae parasitic stages in the abomasum among the sheep breeds (Supplementary information 3). Santa Inês lambs presented the lowest counts of total larvae, early L_4 and late L_4 compared to Texel (p-values = 0.0128, 0.0133 and 0.0089, respectively) and White Dorper (p-values = 0.0077, 0.0306 and 0.0074, respectively) (Fig. 2e, f and g), while similar trend was also observed for total worm counts ($p = 0.0991$) (Fig. 2d). The adult per immature worm ratio presented a trend to be higher in Santa Inês compared to other breeds ($p = 0.0751$) (Fig. 2h).

Regarding the parasitic classification into sexes, both male L_4 and L_5 counts were significantly lower in Santa Inês compared to Dorper (p-values = 0.0037 and 0.0334, respectively), while only L_4 male worms were significantly lower compared to Texel ($p = 0.0095$) (Fig. 2j and k) The female L_4 larvae counts presented a trend to be lower in Santa Inês compared to White Dorper ($p = 0.0639$), while Texel presented intermediated levels with no differences compared to the other breeds (Fig. 2i). The female L_5, female adult and male adult worm counts showed no differences among breeds (Supplementary information 3).

3.4. Relative quantification of gene expression

The results of relative gene expression comparing sheep breeds are presented in Fig. 3 and Supplementary information 4.

Regarding the genes related to innate response, specifically to pattern recognition receptors, *TLR2* was significantly lower in Texel compared to White Dorper ($p = 0.0258$) and Santa Inês ($p = 0.0346$) (Fig. 3a), while *TLR7* was similar among breeds. and the mRNA levels of *TLR4* and *NFBKIA* genes presented a trend to be lower in Texel compared to other breeds (p-values = 0.1418 and 0.0979) (Fig. 3b and c).

The complement activation related *C7* gene was significantly lower in Texel compared to White Dorper ($p = 0.0225$), while similar levels were observed in Santa Inês compared to other breeds (Fig. 3d). The *CFI* mRNA transcripts were similar among the sheep breeds.

The *GAL11* gene was upregulated in White Dorper compared to Texel ($p = 0.0014$), while Santa Inês presented similar levels compared to other breeds (Fig. 3e). *GAL14* mRNA levels presented a trend to be higher in White Dorper compared to Santa Inês ($p = 0.0635$) (Fig. 3f).

The pro-inflammatory related *TNFA* and *IL33* genes were similar among the breeds. *IL10* and *TGF* transcripts presented a trend ($p = 0.0736$ and 0.08) to be elevated in White Dorper (609.78 and 8.60-fold change means) compared to Santa Inês (304.99 and 5.13-fold change means) and Texel (268.76 and 3.09-fold change means) (Fig. 3g and h). While the pro-inflammatory gene *IL1*B, was increased in White Dorper, especially compared to Texel (Fig. 3h). *IL1B* presented no amplification during qPCR assay for Texel animals and for 7 out of 8 Santa Inês lambs, while 6 out of 8 White Dorper lambs were positive for this gene. However, since most lambs presented no amplification for this gene, its relative expression was not calculated, but the results are available in the raw data file.

There were no significant differences for gene expression related to mucin activity (*B3GNT3*, *CLCA1* and *TFF3*) and for some Th2 profile related responses (*IL13* and *IL5*). However, *IL4* and *MS4A2* mRNA levels presented a trend ($p = 0.096$ and $p = 0.121$, respectively) to be higher in Texel (0.49 and 604.97-fold change means, respectively), compared to White Dorper (0.2262 and 293.00-fold change means, respectively) and Santa Inês (-0.5902 and 308.65-fold change means, respectively)

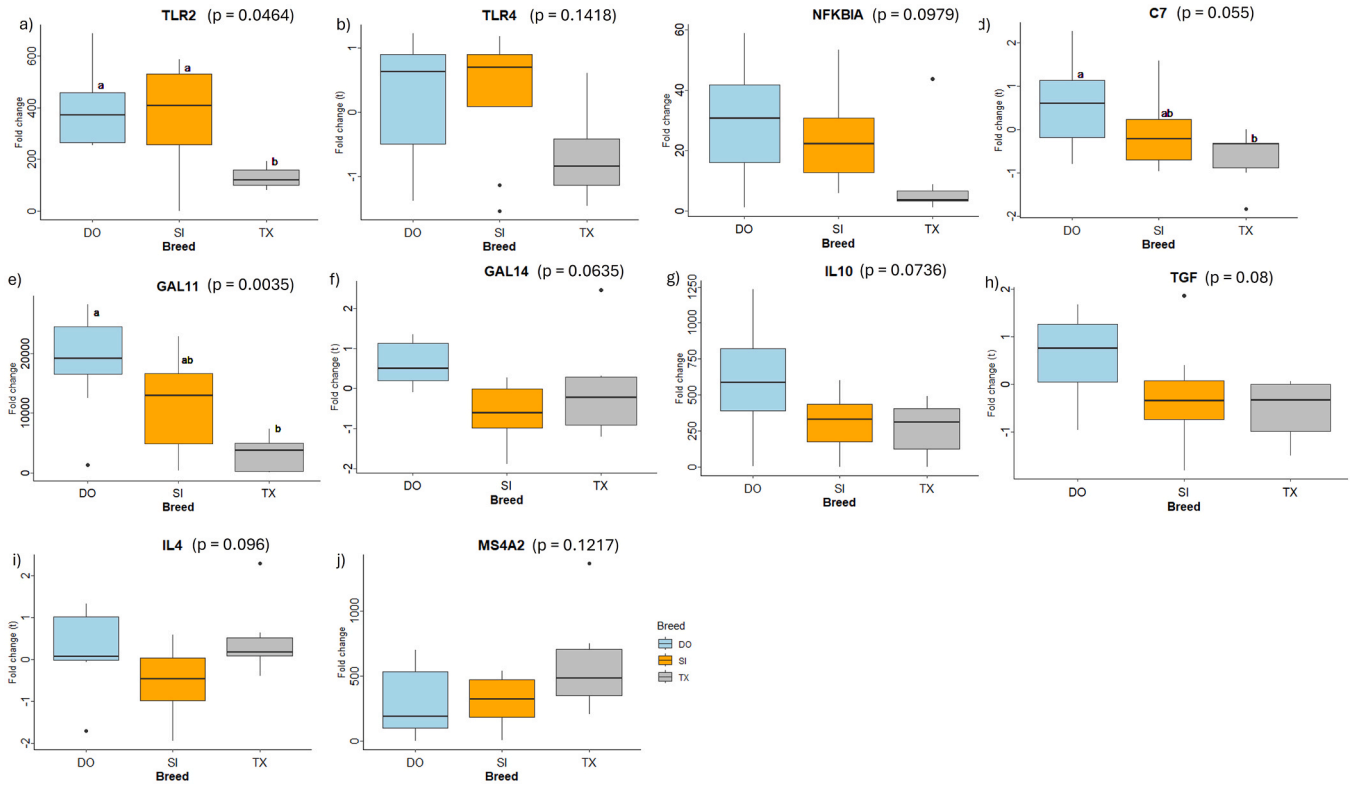

Fig. 3. Relative quantification of gene expression in the fundic abomasum of White Dorper (DO), Santa Inês (SI) and Texel (TX) lambs naturally infected *with H. contortus*. The presented p-values are derived from the ANOVA test. Different letters among the sheep breeds indicated significant differences by Duncan test ($p < 0.05$). The "t" letter in the y-axis title indicated transformed data.

(Fig. 3i and j). Most animals presented no amplification of the *IL13* gene, as only 3 out of 8 White Dorper, 2 out of 7 Texel and 2 out of 8 Santa Inês presented positive amplification. Then, relative expression was not calculated, and the results are available in the raw data file.

3.5. Correlations

3.5.1. Correlation between parasitic stages and red blood cells parameters

The hemoglobin and hematocrit (by complete hemogram method) levels were negatively correlated with all parasitic stage levels (including FEC), with the highest correlation coefficient for total worm counts ($\rho = -0.75$ and $\rho = -0.72$, respectively, $p < 0.01$) (Fig. 4). The hemoglobin levels presented the highest correlation values for all parasitic counts, except for L_4 and male larvae counts which were slightly higher for hematocrit values. The PCV obtained by micro-hematocrit method presented significant negative correlations with male L_5 and early L_4 larvae counts (Fig. 4).

FEC presented the highest positive correlation with female adult worm counts ($\rho = 0.81$, $p < 0.01$), compared to all parasitic stage counts.

3.5.2. Correlations between gene expression levels and parasitic stage counts

The most important significant correlations between gene expression levels, specific larvae stages and sex are presented in Fig. 5, while the correlation among other gene expression levels, parasitic stage counts, and red blood cell related parameters are showed in Supplementary information 5.

TLR2 levels were negatively correlated with L_4 larvae counts ($\rho = -0.46$, $p < 0.05$), male L_4 counts ($\rho = -0.47$, $p < 0.05$), initial L_4 larvae counts ($\rho = -0.60$, $p < 0.01$), while positive correlation was observed for adult/immature ratio ($\rho = 0.52$, $p < 0.05$). The mRNA levels of this gene were also positively correlated with *IL10* ($\rho = 0.50$, $p < 0.05$), *NFKBIA* ($\rho = 0.51$, $p < 0.05$) and *C7* ($\rho = 0.55$, $p < 0.05$), and negatively correlated with *TFF3* ($\rho = -0.46$, $p < 0.05$).

TLR4 transcripts were negatively correlated with all adult worm counts ($\rho = -0.45$ to -0.48, $p < 0.05$), total worm counts ($\rho = -0.47$, $p < 0.05$), FEC ($\rho = -0.42$, $p < 0.05$), L_4 larvae counts ($\rho = -0.43$, $p < 0.05$) and early L_4 counts ($\rho = -0.48$, $p < 0.05$). Negative correlations for this gene were also found with *IL4* ($\rho = -0.56$, $p < 0.01$) and *MS4A2* ($\rho = -0.56$, $p < 0.01$).

TLR7 gene expression was positively correlated with *TGF* ($\rho = 0.52$, $p < 0.05$) and *IL10* ($\rho = 0.47$, $p < 0.05$).

NFKBIA transcripts were positively and strongly correlated with *C7* and *TGF* ($\rho = 0.81$ and $\rho = 0.80$, respectively, $p < 0.001$).

CFI transcripts were negatively correlated with all adult worm counts ($\rho = -0.45$ to -0.50, $p < 0.05$) and with FEC levels ($\rho = -0.47$, $p < 0.05$), while positive correlation was observed with *GAL11* gene expression ($\rho = 0.45$, $p < 0.05$).

C7 mRNA levels were positively correlated with *B3GNT3* ($\rho = 0.51$, $p < 0.05$) and *IL10* ($\rho = 0.53$, $p < 0.05$).

Both *GAL11* and *GAL14* transcripts were positively correlated ($\rho = 0.47$, $p < 0.05$). *GAL11* was negatively correlated with FEC ($\rho = -0.53$, $p < 0.05$) and positively correlated with hemoglobin levels ($\rho = 0.49$, $p < 0.05$). *GAL14* was positively correlated with *IL5* ($\rho = 0.54$, $p < 0.01$) and *TFF3* ($\rho = 0.50$, $p < 0.05$) mRNA levels.

The gene expression of anti-inflammatory cytokines *TGF* and *IL10* was positively correlated ($\rho = 0.62$, $p < 0.01$).

IL33 mRNA levels were positively correlated with total larvae counts ($\rho = 0.44$, $p < 0.05$) and negatively correlated with IL5 ($\rho = -0.59$, $p < 0.01$).

CLCA1 gene expression was negatively correlated with FEC ($\rho = -0.59$, $p < 0.01$). *TFF3* mRNA levels were negatively correlated with PCV values ($\rho = -0.47$, $p < 0.05$).

MS4A2 transcripts were positively correlated with *IL4* expression ($\rho = 0.64$, $p < 0.01$) and negatively correlated with PCV values ($\rho = -0.59$,

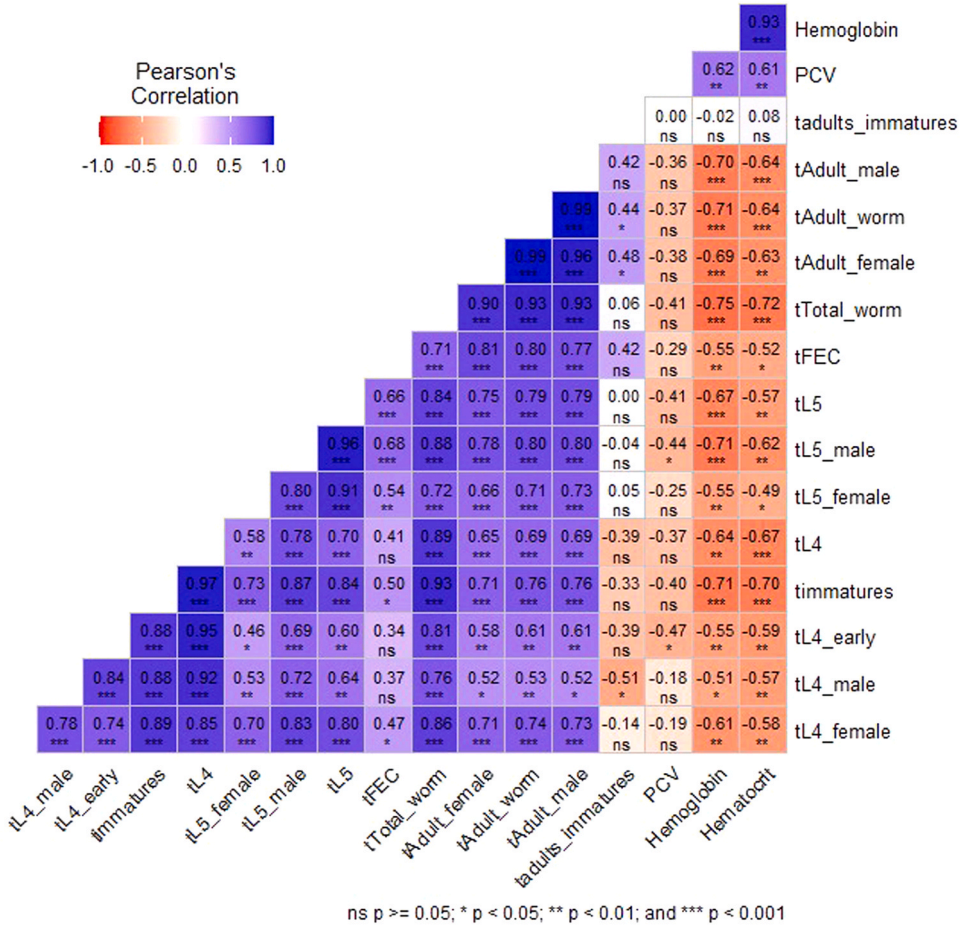

Fig. 4. Correlation by Pearson test among parasitic stage counts (FEC, early L_4, male L_4, female L_4, L_4, male L_5, female L_5, L_5, immatures, male adult, female adult, adult, total worm, adult/immature ratio) and red blood cell parameters (hematocrit and hemoglobin by hemogram, PCV by microhematocrit method).

$p < 0.01$).

4. Discussion

As expected, and similarly to previous studies (Chagas et al., 2024; Kapritchkoff et al., 2024), when evaluating naturally *H. contortus* infected animals, Santa Inês lambs presented improved resistance, presenting a tendency of lower total worm counts in the abomasum, while intermediated resistance was found for Texel, and the most susceptible breed evaluated in our study was White Dorper.

In spite of this observation, female L_5 larvae and adult worm counts were similar among sheep breeds. The early L_4 and L_4 larvae counts were significantly lower in Santa Inês lambs compared to the other sheep breeds. According to Shaw et al. (2012) the IgA-type antibodies present in saliva could be participating in the resistance to *H. contortus*, as high levels of IgA specific to L_3 parasites, and to a lesser extent to L_4, have been determined in animals resistant to infection. Unexpectedly, the adult/immature ratio presented a tendency to be superior in Santa Inês compared to Texel, while White Dorper lambs presented values similar to both breeds.

Higher *TLR2* transcript levels were observed in both Santa Inês and White Dorper lambs compared to Texel, and negative correlations between *TLR2* levels and all L_4 larvae stage counts, especially early L_4 larvae, were found. In this sense, we hypothesize an important role of this pattern recognition receptor (PRR) in the host response during earlier stages of *H. contortus* infection, which allowed the reduced proportion of immature compared to adult worms, mainly in Santa Inês and in lower extent also in White Dorper lambs. In addition, the *TLR4* mRNA levels presented a tendency to be higher in Santa Inês and White Dorper, and negative correlations were observed between this transcript and all adult worm counts, FEC, late L_4 and early L_4 values. This indicates that PRR has a role in the host response to clear the parasite infection. However, contrary to *TLR2*, *TLR4* seems to act on both earlier and latter parasitic stages. The major role of *TLR2* and *TLR4* signaling for eosinophils recruitment and IgA production, involved in the development of effective immune responses, was already observed against larval ascariasis (Nogueira et al., 2021). Higher gene expression of *TLR2* and *TLR4* in the abomasal mucosa were previously associated with the resistant phenotype in Merino sheep challenged with *H. contortus* or *Trichostrongylus colubriformis* (Ingham et al., 2008). In a study evaluating Morada Nova breed experimentally infected with *H. contortus*, *TLR2* was upregulated in the abomasum of resistant lambs compared to susceptible ones, while *TLR4* was similar between those groups (Toscano et al., 2019). Further, in Morada Nova lambs naturally infected with *H. contortus*, homozygous animals for the A haplotype of β-globin (considered as resistant to haemonchosis), presented a trend of higher *TLR2* transcripts compared to B homozygous animals (Okino et al., 2023). Recently, a study in Malpura sheep classified as susceptible and resistant to *H. contortus* infection, demonstrated that transcription profiles of several TLRs, including *TLR2* and *TLR4*, in the peripheral blood were significantly upregulated in resistant sheep when the animals presented similar parasite loads, but down-regulated in resistant animals when the parasite loads in susceptible sheep reached very high levels (Kumar et al., 2024). These last findings, added to our limited number of experimental animals, may explain the absence of significantly differentiated expression profiles for some evaluated genes. The absence of a more pronounced difference in the worm counts among breeds is probably due to the chronic stage of haemonchosis, since lambs

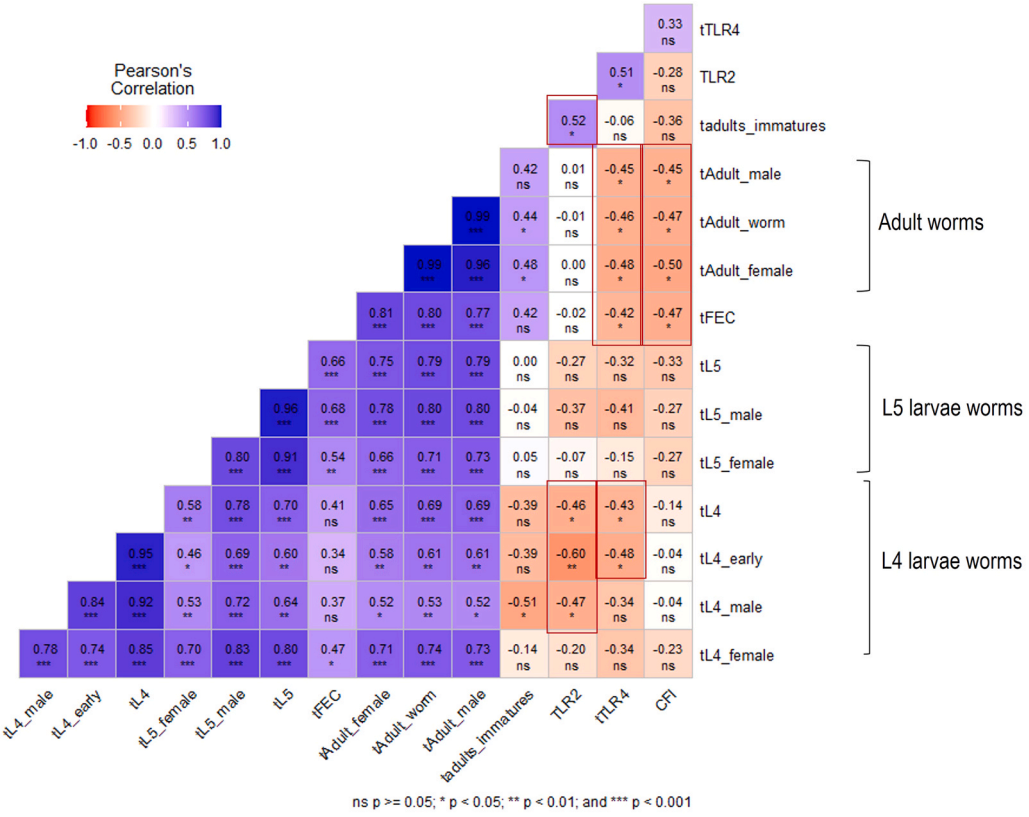

Fig. 5. Correlations by Pearson test among gene expression levels (*TLR2*, *TLR4* and *CFI*), parasitic stage counts (FEC, L_4 larvae, L_5 larvae, adult, adult/immature ratio) classified by sex (female and male).

remained infected from the first monitoring age (63 days) through the euthanasia day, at approximately 210 days of age.

Pathogen-associated molecular patterns (PAMPs) present in parasites and other microorganisms are recognized by TLRs, which will trigger the down-stream pathways involved in adaptive immune responses by activating multiple antigen-presenting cell (APC) functions (Rajasekaran et al., 2017). TLR activation led to activation of mitogen-activated protein kinase pathways (*MAPK*) and consequently translocation of nuclear factor kappa B (*NF-kB*) to nucleus. *NFkB* is activated by phosphorylation of its inhibitory subunit, the *NFKBIA* (or *IkBα*), and induces transcription and production of pro-inflammatory cytokines and stimulation of other numerous genes in response to pathogens (Rajasekaran et al., 2017; Cohen et al., 1998).

In the present study, most inflammatory related genes, including the pro-inflammatory mediators *NFKBIA* and *IL1B*, and anti-inflammatory cytokines *IL10* and *TGF* were upregulated in White Dorper, especially compared to Texel. Upregulation of *NFKBIA* was observed in PBMCs of Boer goats experimentally infected with *H. contortus* (Wang et al., 2024). Similar findings associated increased inflammatory response to more susceptible animals (intra-breed comparison). In Morada Nova lambs infected with *H. contortus*, increased inflammatory response, mediated by *IL1B*, *IL10* and *TNFA*, was detected in susceptible animals, classified by phenotyping or β-globin polymorphism (Toscano et al., 2019; Okino et al., 2023). Additionally, a prolonged chronic inflammatory response in the abomasum of susceptible sheep was described and characterized by elevated expression of *NFKBIA*, *IL10* and *TGFB* (Ingham et al., 2008).

Positive correlations were observed among inflammatory related transcripts, including *NFKBIA*, *TGF*, *IL10*, and other genes which will also lead to inflammation (*TLR2* and *C7*), indicating cascade or co-stimulation, wherein all of them were markedly expressed in the most susceptible White Dorper breed. A pronounced inflammation is expected, since the endoparasites induce injuries in the abomasal mucosa, releasing inflammatory cytokines, accompanied by regulatory activity, mediated by *IL10* and *TGF* cytokines. The *C7* gene encodes a protein which is part of membrane attack complex of classical pathway of complement cascade, responsible for triggering a hole on pathogen surface, leading to cell lysis or death (Sarma and Ward, 2011). Conversely, in a study comparing two sheep breeds from the Canary Islands, presenting different resistant phenotypes, when experimentally primo-infected with *H. contortus*, complement related genes, including *C7* and *CFI*, were significantly upregulated at 20 days post-infection in the resistant breed compared to respective non-infected group, while in the susceptible breed, none of these genes were altered by infection (Guo et al., 2016). These diverging results may be due to the differential phases of disease, which may be considered as acute phase in this last study, but chronic in our experiment, or due to the different parasite loads, as discussed for the TLRs activation by Kumar et al. (2024) (Kumar et al., 2024).

The *CFI* is a crucial inhibitor controlling all complement pathways, through degradation of activated complement proteins C3b and C4b added to other cofactors (factor H, C4b-binding protein, complement receptor 1 or CD46) (Nilsson et al., 2011). *CFI* transcripts were negatively correlated with all worm counts related to adult stages and FEC values, indicating an important role in the host clearance of latter parasitic stages. In our study evaluating specific humoral responses against excretory-secretory antigen of 24 kDa (ES24) of *H. contortus* in naturally infected Morada Nova lambs, the resistant phenotype, associated to female sex and/or A homozygous animals for the β-globin haplotype, presented high levels of anti-ES24 immunoglobulins of IgM isotype, which were negatively and positively correlated with FEC and PCV values, respectively. On the other hand, IgG and IgA isotypes presented no association with resistance (Okino et al., 2024). IgM is able to activate the classical complement pathway with 1000-fold higher avidity than IgG (Cooper, 1985). Important role of specific titers of IgM

isotype and complement fixation were described on the parasitic clearance of *Strongyloides ratti* (Nouir et al., 2012) and *Strongyloides stercoralis* (Brigandi et al., 1996).

The *GAL11* was upregulated in White Dorper compared to Texel. This galectin, expressed by epithelial cells of the gastrointestinal tract after parasite infection, was previously associated with *H. contortus* resistant lambs (Okino et al., 2023). *GAL14*, expressed by eosinophils after allergenic or parasitic stimulation, also trended to be higher in White Dorper compared to Santa Inês and Texel breeds. Both galectins were positively correlated, and *GAL11* levels were closely associated with *H. contortus* resistance, through negative and positive correlations to FEC and PCV values, respectively. While *GAL14* was positively correlated to *IL5* and *TFF3* transcripts. Galectins seem to have a role in the enhancement of mucus adhesiveness and consequently reduction of *H. contortus* motility (Robinson et al., 2011). *TFF3* was negatively correlated to PCV levels, indicating association with clinical disease, and is considered one of the main trefoil factors secreted by normal mucus-secreting cells in the GI epithelium, co-expressed with mucins, and seems to protect mucosal surface integrity (Rinaldi et al., 2011). *CLCA1* is also involved in mucus dynamics and was previously associated with *H. contortus* resistance (Okino et al., 2023; Nyström et al., 2018), *CLCA1* transcripts were negatively correlated with FEC levels in the present study.

There were no significant differences in the Th2 related mediators among sheep breeds. However, *IL4* and *MS4A2* transcripts presented a trend to be higher in Texel, which may explain the slightly superior resistance to haemonchosis compared to White Dorper animals. *IL4* and *MS4A2* showed a significant positive correlation, while *MS4A2* and PCV levels showed a significant negative correlation, as detailed in Supplementary Information 5.. Increased expression of these two Th2 related genes were previously associated with resistance against *H. contortus* (Barbosa Toscano et al., 2020; Okino et al., 2023; Ingham et al., 2008).

5. Conclusion

Decreasing resistance against *H. contortus* infection among sheep breeds was observed in the following sequence: Santa Inês, Texel and White Dorper. Differential local immune responses were developed during chronic infection, wherein both Santa Inês and White Dorper presented high activity of innate receptors, especially *TLR2*. While Th2 related transcripts were superior in Texel lambs. White Dorper animals also presented increased local inflammation. The host responses to different parasite stages were characterized by *TLR2* activity for earlier parasitic stages, while complement activity (*CFI*) seemed to be involved in the clearance of latter parasite stages. Further, *TLR4* activity seemed to be involved in the responses to both early and late stages of this parasite. To our knowledge, this is the first study to point out for differential immune responses among sheep breeds and to different parasitic stages. Given the exploratory nature of present study, future experiments are required to confirm our obtained results. The better elucidation of these host-parasite interactions may lead to the improvement of immune-prophylactic management of haemonchosis in sheep.

CRediT authorship contribution statement

Chagas Ana Carolina de Souza: Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Esteves Sérgio Novita:** Writing – review & editing, Methodology, Investigation. **Minho Alessandro Pelegrine:** Writing – review & editing, Methodology, Investigation. **Niciura Simone Cristina Méo:** Writing – review & editing, Visualization, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Melito Gláucia Roberta:** Methodology, Investigation. **Okino Cintia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bello Hornblenda Joaquina Silva:** Writing – review & editing, Methodology, Investigation. **Campos Emanuelle Martins de:** Methodology, Investigation. **Kapritchkoff Rafaela Tami Ikeda:** Methodology, Investigation. **Cunha Amanda Freitas da:** Methodology, Investigation. **Costa Estevão Camillo da:** Methodology, Investigation.

Authors contributions

CH Okino, SCM Niciura and ACS Chagas designed and conceived the study; CH Okino, HJS Bello, AF Cunha, EC Costa, EM Campos, RTI Kapritchkoff, GR Melito and AP Minho performed the experiments in the field and in the laboratory (parasitological and molecular assays); SN Esteves was responsible for nutrition and managements of the lambs; CH Okino and SCM Niciura analysed the data; CH Okino wrote the manuscript; and SCM Niciura, HJS Bello, ACS Chagas and AP Minho revised critically. Funding was conceded to ACS Chagas. All authors discussed the results and contributed to the final manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2025.110936.

Data availability statement

All data were inserted as raw data file, other information was included as supplementary information 1–4. Additional information related to this study will be available if requested.

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