Early-onset immune response to *Haemonchus contortus* infection in resistant Santa Ines suckling lambs compared with susceptible Ile de France

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Abstract: Santa Ines, an indigenous Brazilian hair sheep, in comparison with European sheep breeds (e.g., Ile de France), show greater resistance against gastrointestinal nematode (GIN) infections, mainly to *Haemonchus contortus*. Here we bring novel findings that address some gaps regarding the resistance traits involved in the development of the immunity of young lambs in the first few weeks of life to *H. contortus* infection. This study aimed to compare parasitological, humoral, and local effector cell-mediated responses, also the histopathological differences in the abomasal mucosa of Santa Ines (SI) and Ile de France (IF) suckling lambs serially infected with *H. contortus*. Parasitological variables, local and circulating humoral immunity, and local cellular response were evaluated in naïve Santa Ines (n=14) and Ile de France (n=12) lambs, randomized into four groups: infected SI (n=8), non-infected control SI (n=6), infected IF (n=8) and non-infected control IF (n=4). Lambs from infected groups were first infected at 14 days old, and multiple infections were conducted every second day, until the age of 66 days old (52 days post first infection). In comparison with infected Ile de France, infected Santa Ines lambs had lower mean eggs per gram of faeces, lower total *H. contortus* worm burden, lower females' length, greater abomasal lymph node weight, greater mucosal thickness in the fundus, and also higher counts of eosinophils in the fundus, and mast cells and globule leukocytes in both fundic and pyloric mucosa of the abomasum. Intrabreed differences were observed into the infected Santa Ines group, with three of the eight lambs classified as highly resistant for displaying *H. contortus* burden ranging only from 1 to 42 worms. Overall, Santa Ines suckling lambs showed great resistance against *H. contortus* infection in comparison with Ile de France lambs, being able to mount a robust innate immune response at an early age, and before weaning.

Keywords: Effector cells, Innate response, Local immune response, Nematode, Resistance.

1. Introduction

Gastrointestinal nematode (GIN) infections are responsible for high economic losses to the small ruminant industry globally. *Haemonchus contortus* is the main gastrointestinal parasite of small ruminants in tropical and subtropical areas worldwide, and haemonchosis prophylaxis based on anthelmintics has been shown to be unsustainable due to the widespread appearance of nematode populations resistant to all available classes of anthemintics (Almeida et al., 2010; Amarante, 2014; Albuquerque et al., 2017).

Against this background, the development of alternative control for GIN infections, such as nutritional supplementation (Bricarello et al., 2005; Carvalho et al., 2015), vaccination (Bassetto et al., 2020; Britton et al., 2020) and the breeding for resistance (Amarante et al., 2009; Shakya et al., 2009), have emerged as promising outlooks. Santa Ines sheep, a Brazilian indigenous hair breed, has been reported as resistant to *H. contortus* at different age categories, when compared to commercial European sheep breeds (Amarante et al., 2004; Rocha et al., 2005; Amarante et al., 2009; Rocha et al., 2011; Albuquerque et al., 2019).

Gastrointestinal nematode infections are often associated with type 2 immunity, and over the last few years several investigations have demonstrated that genetic resistance of the host to overcome GIN infections varies according to the age category, breed, individual variation within the same breed, and also physiological condition of the host (Bricarello et al., 2005; Amarante, 2014; Shakya et al., 2011; McRae et al., 2014; Jacobs et al., 2016; Patra et al., 2016). Resistant animals tend to present more pronounced and earlier cellular and/or humoral Th2 response (McRae et al., 2015; Inclan-Rico and Siracusa, 2018). Under a Th2 mediated environment, Th2 associated cytokines (such as IL-4, IL-5, IL-9, IL-10, and IL-13) induce the hyperplasia and migration of activated effector cells (such as eosinophils and mast cells) to the site of infection (McRae et al., 2015; Aboshady et al., 2020).

A number of studies have identified sheep breeds resistant or susceptible to *H. contortus* infection (Bricarello et al., 2005; Shakya et al., 2011; Jacobs et al., 2016; Patra et al., 2016), however, the immunological mechanisms that confer greater resistance to the animals and the age of onset have not yet been completely elucidated. To our knowledge, there are only few studies that evaluate the mucosal immune response of naïve suckling lambs in the first few weeks of life against *H. contortus* infection (Emery et al., 2000).

By addressing the current understanding of the early age of sheep to mount an adequate onset immune response to *H. contortus*, our data bring novel findings regarding the mechanisms involved in the innate immunity, and contribute to the development of better strategies for the very early selection of resistant animals. In this study we compared parasitological, humoral, and effector cell-mediated responses, also the histopathological differences in the abomasal mucosa of resistant Santa Ines and susceptible Ile de France suckling lambs serially infected with *H. contortus*.

2. Material and Methods

The study was conducted in the experimental area of the Bioscience Institute (IBB) UNESP - Botucatu, SP, Brazil. All the procedures involving animals in this study were in accordance with the local Ethics Committee on Animal Use (protocol number 0118/2018, FMVZ-UNESP).

2.1. Third stage (L3) larvae production

Two donor lambs were drenched on arrival with the following combination of anthelmintics: albendazole (20 mg/kg of body weight (BW), Valbazen®, Pfizer), levamisole (9.4 mg/kg of BW, Ripercol®, Zoetis), and monepantel (2.5 mg/kg of BW, Zolvix®, Novartis) for three consecutive days. Then, the worm-free status of the donors was confirmed

by a series of parasitological examination of individual faecal samples. Afterwards, both animals were artificially infected orally with 5,500 *H. contortus* L3 larvae in a single dose, using an *H. contortus* isolate with susceptibility to all anthelmintic classes (Echevarria et al., 1991). At least 21 days after infection, donor lambs' faeces were collected individually into collecting plastic bags twice a day, and cultured for one week under ideal conditions of humidity and temperature (Ueno and Gonçalves, 1998) in a BOD (Biological Oxygen Demand) incubator. Third stage larvae (L3) were harvested and stored at 4 ºC in deionized water, and used, up to a maximum of seven days after recovering to orally infect the experimental suckling lambs.

During the trial, donor lambs were housed indoors, with free access to potable water and mineral salt (OvinoFós Tortuga®), and were fed *ad libitum* with *Cynodon* spp. hay free of nematode-infective larvae.

2.2. Animals and experimental design

The experimental design has been previously described (Lins et al., 2020). Briefly, naïve lambs were born in the experimental area, and after birth each ewe and its respective lamb were kept in covered stalls, with concrete floors, feeders and water fountains, that were washed daily in order to prevent undesired parasitic infections. During the experimental period, ewes and their lambs had free access to potable water and mineral salt (OvinoFós Tortuga®), were fed with *Cynodon* spp. hay free of nematode-infective larvae, and received a daily dietary supplement with 18% of crude protein (Nutrição Animal Coopermota®) in an amount that corresponded to 2.5% of the BW of the ewes, and offered two times a day.

A 2 x 2 factorial design was used, with two infection status (infected and non-infected control) and two sheep breeds (Santa Ines and Ile de France). Naïve Santa Ines (n=14) and Ile de France (n=12) suckling lambs, 14 days old, were randomized into four groups according

to the order of birth: infected Santa Ines (n=8), non-infected control Santa Ines (n=6), infected Ile de France (n=8) and non-infected control Ile de France (n=4) (Figure 1A). Lamb groups within the same breed had similar weigh at the day of birth and at the beginning of the infection protocol (14 days old; day of first infection) (Table S3).

2.3. Experimental infection

Naïve suckling lambs were artificially infected every two days, with third stage larvae (L3) of *H. contortus* directly into the oral cavities. Experimental infections were divided into three steps, differing in the number of L3 administered at each stage. The lambs were first infected at 14 days old, and infection lasted until 66 days old. From 14 to 30 days old, the lambs received 900 L3, divided in nine artificial infections with 100 L3 each; from 32 to 48 days old, the lambs received 1800 L3, divided in nine artificial infections with 200 L3 each; and from 50 to 66 days old, the lambs received 2700 L3, divided in nine artificial infections with 300 L3 each. At the end of the infection protocol, 27 experimental infections had been conducted, and each lamb received a total of 5400 L3 (Figure 1B). Non-infected control groups were kept worm-free during the whole trial.

Two days after the last infection (68 days old), lambs were euthanized under sedation with an association of ketamine (4.5 mg/kg of BW, Cetamin®, Syntec) and xylazine (0.05 mg/kg of BW, Calmiun®, Agener União), administered intravenously. Immediately after sedation and confirmation of lack of awareness, exsanguination was performed through jugular and carotid sections.

2.4. Parasitological analyses and blood sample collection

Faecal egg counts (FECs) were performed every six days, starting on 0 day of the first infection (14 days old) and lasting until 54 days post first infection (d.p.i.), when lambs were

68 days old, totalling 10 sampling data collection. Faecal samples were collected directly from the rectum of animals, conditioned in polyethylene bags, and kept refrigerated until the moment of processing. FECs were determined using a modified McMaster technique in which each worm egg counted represented 100 eggs per gram (EPG) of faeces (Ueno & Gonçalves, 1998). When EPG was zero, Willis simple flotation, a more sensitive technique, was performed (Willis, 1921).

Additionally, faecal cultures were performed for each lamb individually on the last sampling to confirm that they were not infected by other nematodes. L3s obtained from cultures of infected lambs were identified according to descriptions of Ueno and Gonçalves (1998).

After euthanasia, abomasums were removed, opened along the greater curvature and the contents collected into containers for worm recovering. Then, the abomasums were soaked in 320 mL of saline solution (0.9% NaCl) at 37 °C for 6 h. A 10% aliquot of each content and 100% of the digested material were collected and set in different containers, and then frozen at -20 °C. The remaining 90% aliquots of contents were preserved frozen at -20 °C as backup. All nematodes presented in 10% aliquots and 100% of the digested material were counted, parasites were morphologically identified according to their developmental stage (Ueno and Gonçalves, 1998), and then stored in glass vial bottles with 70% ethanol. Additionally, twenty recovered adult female of *H. contortus* from each animal (infected groups), excepting for the three Santa Ines lambs in which two of them did not present adult females and one presented only one adult. Females of *H. contortus* were randomly picked for total length measurement in millimetres (mm) using a stereomicroscope.

Jugular blood samples (5 mL) were collected into plain tubes containing anticoagulant (Vacutainer® K2 EDTA 7.2mg, BD, Brazil) every six days, also starting at 0 d.p.i. (14 days old) until 54 d.p.i. (68 days old), totaling 10 sampling data collections. Plasma samples were

obtained through blood centrifugation for 15 min/590 g at 4 ºC. After separation, plasma samples were stored at -80 °C until use in immunoglobulin G (IgG) antibody measurements against antigens of *H. contortus* L3 by Enzyme-linked immunosorbent assay (ELISA).

2.5. Abomasal and lymph node tissue collection

After opening the abomasums, two pieces (2cm x 2cm each) were collected (one from fundus and one from pylorus) and quickly stored at -20 °C until processing for mucus extractions, to determine the parasite-specific levels of immunoglobulin A (IgA) against antigens of *H. contortus* L3. Tissue samples of the abomasum were also collected for histological procedures (2cm x 2cm each, one from fundus and one from pylorus). Abomasal lymph nodes were collected and weighed.

2.6. Histopathological procedures and cell counting

The abomasal fundus and pylorus tissue samples were set into buffered formalin solution (4%) and fixed for eight hours. Then, formalin solution was removed and 70% ethanol was added. Samples were kept at 4 °C until embedded in paraffin wax for routine histological procedures. Tissue sections of abomasums (5 µm thick) were stained with toluidine blue (1%), Haematoxylin and Eosin (H&E). Eosinophils and globule leukocytes were counted on H&E stained sections, whereas mast cells were counted on sections stained with 1% toluidine blue. Eosinophils and mast cells were counted in the mucosa, muscularis mucosae and submucosa regions separately, while globule leukocytes were counted only in the abomasal mucosa. All the cells were counted in the fundic and pyloric regions.

The protocol applied for eosinophil, mast cell and globule leukocyte counts on the mucosa was described by Scott et al. (2017) with some modifications: eosinophils, mast cell and globule leukocyte in areas where the fundic and pyloric glands were orientated in

194 longitudinal section, were counted at x1000 magnification, using a 1 cm^2 eyepiece graticule.
195 Counts were made at five locations in each section in a 250 µm wide column of mucosa from
196 the muscularis mucosae to the luminal surface. Regarding the muscularis mucosae and sub-
197 mucosa regions, eosinophils and mast cells were counted in thirty randomly selected fields of
198 view per animal at x1000 magnification, in 100 μm^2 (in the muscularis mucosae) and 2500
199 μm^2 (in the sub-mucosa) areas (adapted from Balic et al., 2000b).
200 Additionally, fundic and pyloric tissue sections were stained with Periodic acid-
201 Schiff (PAS) for all mucins (carbohydrate residues) (Simpson et al., 2016). Firstly, all PAS-
202 stained sections were evaluated (infected and non-infected control groups) to define the
203 intensity of Periodic acid-Schiff positive reactivity, then a qualitative classification
204 (weak, moderate or strong staining) was performed. PAS-positive material was evaluated in
205 sections from the basal zone to the luminal surface in five randomly selected locations of
206 view at x100 magnification.
207 Mucosal thickness of pyloric and fundic regions was measured directly at two
208 locations in each of two separate sections, from the muscularis mucosae to the luminal
209 surface (Scott et al., 2017), and results were expressed in micrometres (µm).
210
211 ***2.7. Enzyme-linked immunosorbent assay (ELISA)***
212 Plasma samples obtained at four time points (0, 18, 36 and 54 d.p.i) were used to
213 estimate IgG antibodies against PBS-soluble extract of *H. contortus* L3. The production of *H.*
214 *contortus* L3 antigens was previously described by Amarante et al. (2009) and determination
215 of parasite-specific plasma IgG levels was previously described by Silva et al. (2012), with a
216 few modifications: the plates were coated with 2 µg of antigen/mL; each washing was done
217 three times, rotating through 180° and re-washing three more times. Plasma sample from a
218 worm free animal was used as a negative control (NC), as previously described by Santos et

al. (2014), while the plasma positive control (PC) sample was from a naturally trichostrongylid infected lamb described by Albuquerque et al. (2019). Plasma samples were diluted (1:400) in PBS-GT (0.1% Gelatin and 0.05% Tween 20 in phosphate-buffered saline 7.2), and the secondary antibody was diluted at 1:40,000. Results were presented as the percentage of optical density value (OD) of the PC plasma (Kanobana et al., 2001).

Mucus was collected by slightly scraping the mucosal surface with a glass slide, and scrapings were collected into Falcon tubes which were kept on ice. Phosphate-buffered saline supplemented with protease inhibitors (Complete Mini Solution1®, Roche, USA) were added to each sample using a 1:3 dilution. The tubes were manually shaken for 1 h at 4 °C and centrifuged for 30 min/3000 *g* at 4 °C. Supernatant was separated and centrifuged for 30 min/15000 *g* at 4 °C.

IgA levels in abomasal mucus were determined as described by Silva et al. (2012), with some modifications: the plates were coated with 2 µg of antigen/ml; each wash was done three times, rotating through 180° and re-washing three more times; mucus samples were diluted in PBS-GT (1:20) and rabbit anti-sheep IgA peroxidase-conjugated antibody was diluted at 1:80,000. IgA positive control sample (animal 7, OD mean 0.668) was from a naturally trichostrongylid infected lamb described by Albuquerque et al. (2019). The results were expressed as the OD value minus OD blank sample (Kanobana et al., 2001).

2.8. Statistical analysis

Data were evaluated in a normality test (Shapiro-Wilk test), and worm burden, lymph node weight, female *H. contortus* length, and mucosal thickness had normal distribution. All the other variables were transformed to log10(x + 1) prior to analysis. Furthermore, data were analyzed by analysis of variance through the General Linear Model (GLM) for the variables measured just once and at several time points. Group means were compared by Tukey's test

at a 5% significance level, and only significant effects and interactions were reported in the results. Means are presented in the results as the arithmetic means (± standard error). PAS-positive staining data were analysed by frequency analysis using the Fisher's exact test at 5% level of significance. All statistical procedures were conducted through the Statistical Analysis System (version 9.2; SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Parasitological differences between breeds

The *Haemonchus* EPG means of the infected groups are shown in Figure 2A. *H. contortus* eggs were detected for the first time 24 d.p.i.. FEC means increased in both infected groups over the time, however, the increase was more pronounced in the infected Ile de France lambs with significant differences between group means on the last two samplings. All infected Ile de France lambs were shedding eggs on faeces at the end of the trial (54 d.p.i.). Importantly, three lambs of the infected Santa Ines group (animals 1, 2 and 4) stopped shedding eggs over the experiment (Figure S1).

Only *H. contortus* L3 were obtained from cultures of infected groups. Lambs from non-infected control groups did not shed eggs in faeces during the experiment, L3s were not recovered from their faecal cultures, and no worms were found in their abomasums contents at the end of the study (54 d.p.i.), confirming their worm-free status.

The *H. contortus* L3 establishment rate was higher in Ile de France (22.9%) than in Santa Ines lambs (11.1%). Infected Ile de France had higher worm counting (Figure 2B and Table S1) with significant breed difference ($P<0.05$) for the female early L5, male early L5, adult female, adult male and total worm burden.

An infected Santa Ines lamb had only one female worm recovered, and two infected Santa Ines lambs had no females, while all infected Ile de France lambs presented adult

269 females. *H. contortus* females from Ile the France lambs presented greater (P<0.001) length
270 mean (21.55 ± 0.2 mm) than females from Santa Ines lambs (18.65 ± 0.2 mm).
271
272 ***3.2. IgG antibody levels in Santa Ines and Ile de France breeds***
273 Measurement of plasma IgG against *H. contortus* L3 antigens (Figure 3) showed a
274 time effect (P<0.001). The highest IgG levels in both breeds were detected at the beginning
275 of the trial indicating that this immunoglobulin was transferred from ewes to lambs through
276 the colostrum intake. Although infected Santa Ines lambs had higher IgG levels than non-
277 infected Santa Ines lambs, there was no statistical difference between the Santa Ines groups.
278 Similarly, there was no difference in anti-L3 IgG level between infected and non-infected Ile
279 de France lambs. Levels of *H. contortus* L3-specific IgA in abomasal mucus of infected and
280 non-infected control groups were similar to OD blank values, and means were not presented
281 in the results.
282
283 ***3.3. Abomasal responses***
284 ***3.3.1. Lymph node weight***
285 There was a significant breed x infection status interaction (P=0.037) on weight of the
286 abomasal lymph nodes, with the infected Santa Ines lambs presenting the highest average
287 (Figure 4) in comparison with the other groups.
288
289 ***3.3.2. Mucosal thickness and aspect***
290 Infected groups of both breeds had greater mucosal thickness in the fundus and
291 pylorus than their non-infected control group counterparts. Infected Santa Ines lambs had the
292 greatest mucosal thickness in the fundus (P<0.001) of the abomasum, while the

293 measurements of the pyloric mucosa were similar (P>0.05) in infected lambs of both breeds
294 (Figure 5A).

295 In general, mucosal surface of infected Ile de France lambs (Figures 5D and 5I) had
296 more irregularities than infected Santa Ines lambs (Figures 5B and 5G), such as
297 vacuolization, disruption and desquamation of the surface epithelium, submucosa oedema
298 and structural changes in the crypt's morphology. Gastric pits in the fundus and pylorus of
299 non-infected lambs were short and clearly delimitated, mucosal surface was smooth and
300 tissues appeared normal (Figures 5C, 5E, 5H and 5J) with no apparent difference between
301 breeds.

302

303 ***3.3.3. Abomasal cellular responses***

304 Inflammatory cell infiltration was significantly more pronounced in infected Santa
305 Ines lambs, with more marked infiltrate of lymphocytes and eosinophils, mainly at the
306 mucosal base, in comparison with the infected Ile de France lambs.

307 Infected Santa Ines lambs had significantly (P<0.05) greater eosinophil, mast cell and
308 globule leucocyte counts per column in the mucosal of the fundic abomasal region in
309 comparison with the infected Ile de France. Similar results were observed in the mucosal of
310 the pylorus (Table 1) with statistically significant differences in values of mast cells and
311 globule leucocytes. Non-infected lambs of both breeds did not present globule leukocytes in
312 the randomly selected columns, and had a similar small number of eosinophils and mast cells
313 in the fundic and pyloric mucosal tissues.

314 There were no statistical differences between breeds with regards to eosinophil and
315 mast cell counts in the muscularis mucosae of the fundic and pyloric regions. Infected Santa
316 Ines lambs had higher counts of eosinophils and mast cells in the sub-mucosa in comparison
317 with infected Ile the France lambs, regardless of the abomasal region. Controls of both breeds

had no eosinophils in the muscularis mucosae and sub-mucosa, no mast cell in the muscularis mucosae, and presented small numbers of mast cells in the sub-mucosa (Table 1).

PAS reactivity was observed in the middle towards the edge of the mucosae surface in the fundus, and from the basal zone until the luminal surface in the pylorus of infected lambs of both breeds (Figure 6). Most of infected Ile de France lambs had a weak PAS reaction in the fundic folds and a moderate reaction in the pylorus, while most of Santa Ines lambs had a moderate PAS reaction in the fundus and a strong reaction in the pylorus (Table S2). There was no significant difference with regards to PAS reactivity, between infected and non-infected Ile de France lambs. In contrast, PAS reactivity was stronger in fundic and in the pylorus tissues of infected Santa Ines lambs ($P<0.05$) in comparison with their control counterparts.

3.4. Intrabreed differences

Among the infected Ile de France lambs there was one lamb which was slightly more resistant than the other seven. This lamb started shedding eggs later, at 62 days of age (48 d.p.i.), and it had the lowest establishment rate (12.1%, 653 worms) (animal 23, Figure S2).

We found two distinct Santa Ines groups: one more resistant group with three lambs that presented very low worm establishment rate (0.37%, 0.02% and 0.78% in lambs 1, 2 and 4, respectively) with *H. contortus* burden ranging only from 1 to 42 worms; and a less resistant group with five lambs that displayed higher infection intensities, with high EPG ranging from 1200 to 7900 at the end of trial (Figure S1) and worm burdens ranging from 496 to 1284 parasites (worm mean establishment rate of 17.46%). Despite the small number of animals per group, there were consistent significant statistical differences for most of the abomasal variables presented in Table 2. Of the 16 variables analysed at the two regions of the abomasal mucosa, in 14 of them the more resistant group showed values significantly

higher than the less resistant group. With regards to abomasal lymph node weight (mean in gram ± standard error), the less and the more resistant groups had similar means (respectively, 4.89 ± 0.60 and 4.52 ± 1.04). Interestingly, the two infected Santa Ines lambs (lamb 1 and 4) which had strong PAS staining were among those classified as more resistant.

4. Discussion

Previous studies have shown that even in more susceptible categories, as lambs post weaning and periparturient ewes, Santa Ines have higher resistance to *H. contortus* infection in comparison with Ile de France sheep (Amarante et al., 2004; Rocha et al., 2011; Albuquerque et al., 2019). Our results demonstrated that such differences can be seen also in very young lambs, at their first weeks of life making possible to identify the resistant animals precociously. There is lack of information about the immunological mechanism related to worm clearance in resistant suckling lambs, and to address this gap, the present study was the first to examine in detail the resistance and immune response of lambs at an early age. It was noteworthy that among the eight infected Santa Ines, three lambs showed a very robust immune response that practically cleared the *Haemonchus* infection. Therefore, among the Santa Ines, there were lambs able to prevent *H. contortus* parasitism at an age earlier than has been reported for the development of competent immune response against GIN in most commercial sheep breeds (Greer and Hamie, 2016).

The artificial serial infections applied in our trial tried to mimic the natural infection. With the progressive increase in numbers of L3 given to lambs, there was a corresponding increase in the number of worms reaching maturity, and as a consequence, the EPG averages increased over the samplings, mainly in the infected Ile de France lambs. Besides differences between breeds, there was also notable intrabreed differences. The resistance to GIN is a genetically controlled trait that may be affected by age, breed and health status (McManus et

368 al., 2014; Zvinorova et al., 2016) with individual immune response variation among the
369 individuals of the same population (Cardia et al., 2011; Escribano et al., 2019; Cruz-Tamayo
370 et al., 2021). Even in breeds considered susceptible, there are individuals with higher capacity
371 to develop immunity against parasites.

372 The development of the host immune response against *H. contortus* infection requires
373 a continuous challenge that may impair or even prevent the establishment of incoming larvae.
374 Other manifestations of the immune response include arrested worm development, reduction
375 in size, fecundity and elimination of the adult worms (Balic et al., 2000a; Shakya et al., 2011;
376 Santos et al., 2014). Usually, the immune response does not cause the complete elimination
377 of the helminths. Even resistant animals may harbour few worms although with a much lower
378 infection intensity than susceptible animals (Alba-Hurtado and Muñoz-Guzmán, 2013), as
379 observed in the three resistant Santa Ines lambs.

380 Both innate and acquired immunity are associated with the response to nematodes
381 (Mravčáková et al., 2021). However, our results indicated that humoral immune response
382 (IgG and IgA) were not involved in protection against *H. contortus* in the Santa Ines and Ile
383 the France suckling lambs. Both breeds did not produce and release detectable levels of anti-
384 parasitic immunoglobulins, possibly because the period of infections was short. IgG detected
385 in this experiment was possibly related to the transference of maternal immunoglobulin by
386 colostrum (Pfeffer et al., 2005). For this reason, IgG levels decreased over time because this
387 passive humoral response is not long-lasting immunity (ibidem). Effectiveness of humoral
388 immune response, throughout IgG and IgA production against *H. contortus* antigen may be
389 prominent in weaned lambs and older sheep, after primary infections (Shakya et al., 2011;
390 Bowdridge et al., 2013; Hernández et al., 2016).

391 Our data support that the strong local immunity mediated by abomasal effector cells
392 play an important role in development of early defense against *H. contortus* infection. A

393 greater immune-mediated cell hyperplasia in the abomasal lymph nodes occurred in the Santa
394 Ines in comparison with Ile de France lambs. Similarly, in St. Croix hair lambs total
395 abomasal lymph node weight increased exponentially from 2.60 g at day 0 to 6.57 g by day 7
396 following *Haemonchus* infection, whereas the weight only marginally increased in wool
397 lambs (Bowdridge et al., 2015).

398 Besides increase in abomasal lymph node size, the nematode infection induced
399 hyperplasia and inflammatory changes, especially in the abomasal mucosa of Santa Ines
400 lambs, which were more pronounced in the three more resistant lambs. Likewise, in
401 comparison with uninfected animals, *T. circumcincta* infection caused generalized thickening
402 of the abomasal mucosa in sheep (Anderson et al., 1981; Scott et al., 1998). The difference
403 between breeds in relation to mucosal thickness observed in our study was possible due to
404 more intense local inflammatory conditions in the infected Santa Ines, that resulted in high
405 mucosal infiltrate of eosinophils, mast cells, globule leukocytes, lymphocytes, mucous cells,
406 and probably hyperplasia of other cells, such as tuft cells, that are also involved in the
407 immune response (Albuquerque et al., 2019).

408 Mast cells and globule leukocytes are closely linked to the defense against GIN
409 through a strong inflammatory response that can be initiated by the nonspecific degranulation
410 of mast cells and release of vasoactive amines, further activation of the alternative
411 complement pathway and also presence of complement-derived peptides (Huntley et al.,
412 1992; Balic et al., 2002; Balic et al., 2006; Kemp et al., 2009).

413 Gastrointestinal nematode infection induces transcription factors and cytokines
414 production that stimulate stem cells of bone marrow into eosinophil differentiation (Park and
415 Bochner, 2010), increasing significantly the number of eosinophils in the blood (Tizard,
416 2014). The lambs of both breeds of the present trial showed similar numbers of blood
417 eosinophil (Lins et al., 2020), however, the histological analysis showed greater number of

418 eosinophils in the abomasal mucosa of the Santa Ines lambs. It is known that following entry
419 into circulation, eosinophils are recruited and rapidly migrate to tissues at the site of infection
420 (Anthony et al., 2007) in the presence of vasoactive and chemotactic peptides, and in the
421 absence of specific antibodies, can demonstrate cytotoxicity against larvae in early infection
422 stages mediated by complement activation (Kemp et al., 2009). Therefore, eosinophils play
423 important role in the mechanism for a rapid rejection response against incoming L3 *H.*
424 *contortus* (Rainbird et al., 1998; Meeusen et al., 2005).

425 Mucous-producing epithelial cells physiologically release mucus onto the abomasal
426 surface to prevent digestion of the mucosa by gastric juices, and it is considered the first line
427 of defense to the external pathogens, crucial to the protection of the underlying mucosa (Balic
428 et al., 2000a). It has been demonstrated that goblet cells are important effector cells involved
429 in the mucus production in the intestines, and that their hyperplasia is recognized as an active
430 immune response in gastrointestinal nematode infection, however, the abomasum of
431 ruminants does not contain goblet cells and mucus is secreted by surface mucous cells and
432 mucous neck cells (Balic et al., 2000a; Simpson et al., 2016). In the present study, *H.*
433 *contortus* infection in Santa Ines lambs induced surface mucous cells and mucous neck cells
434 in the mucosa of the abomasums, and consequently, increase in secretion of mucus and
435 mucins onto the abomasal surface. Likewise, Simpson et al. (2016) and Mravčáková et al.
436 (2021) showed that *H. contortus* artificial infection in sheep resulted in a strong staining
437 (PAS-positive material) for all mucins throughout the thickness of the abomasum mucosa, as
438 a consequence of a more intense mucus production, mainly by surface mucous cells.
439 Additionally, increases in mucus production generally occur associated with mast cell
440 hyperplasia after nematode infections (Balic et al., 2000a) as observed in our study.

441 In conclusion, our study has brought to light a better understanding of the immunity
442 mechanism against *H. contortus* in Santa Ines suckling lambs that at an early age manifested

a more efficient innate immune response against *H. contortus* in comparison with Ile de France lambs. The resistant lambs presented heavier lymph nodes and increase in the mucosal thickness associated with increase of inflammatory cell infiltration in the abomasal tissues. We also demonstrated the possibility of identification of resistant animals in the first weeks of life, an approach can be useful in programs design for selecting worm-resistant sheep.

Conflicts of interest

The authors declare no conflicts of interest regarding the publication of this paper.

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624

625 **Legends to figures**

626

627 **Figure 1.** Experimental design (1A) and infection (1B). At D68*, lambs were euthanatized.

628

629 **Figure 2. (A)** Averages (± standard error) of eggs per gram of faeces (EPG) and (B) worm
630 counting of the Ile de France and Santa Ines suckling lambs experimentally infected with
631 *Haemonchus contortus*. L4 = fourth stage larvae and L5 = fifth stage larvae. * Means
632 statistically different ($P < 0.05$).

633

634 **Figure 3.** Percentages of Optical Density (± standard error) of IgG against *Haemonchus*
635 *contortus* L3 of infected and non-infected Santa Ines and Ile de France suckling lambs
636 artificially infected with *Haemonchus contortus*.

637

638 **Figure 4.** Abomasal lymph nodes weight of the Ile de France (IF) and Santa Ines (SI)
639 suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control.
640 The ends of the box are the upper and lower quartiles; the median is marked by a horizontal
641 line inside the box; and the two lines outside the box extend to the highest and lowest
642 observations. Different lower case letters are significantly different by Tukey test ($P<0.05$).

643

644 **Figure 5.** Abomasal fundic (**A**) and pyloric (**F**) mucosal thickness (µm) of Ile de France and
645 Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-
646 infected control. The ends of the box are the upper and lower quartiles; the median is marked
647 by a horizontal line inside the box; and the two lines outside the box extend to the highest and
648 lowest observations. Different lower case letters indicate significant difference by Tukey test
649 ($P<0.05$). Photographs of **B**: fundus of infected Santa Ines; **C**: fundus of non-infected Santa

650 Ines; **D**: fundus of infected Ile de France; **E**: fundus of non-infected Ile de France; **G**: pylorus
651 of infected Santa Ines; **H**: pylorus of non-infected Santa Ines; **I**: pylorus of infected Ile de
652 France; **J**: pylorus of non-infected Ile de France. Black arrows: disruption and desquamation
653 of the surface epithelium; Red arrows: vacuolization; Blue arrows: structural changes; Green
654 arrows: submucosa oedema.
655
656 **Figure 6**. Abomasal tissue sections of Santa Ines and Ile de France suckling lambs
657 experimentally infected with *Haemonchus contortus* and non-infected control, stained with
658 Periodic Acid Schiff (PAS) for all mucins. Colour references for qualitative classification:
659 strong (A, D, G, J), moderate (B, E, H and K) or weak (C, F, I and L) staining.
660

661 **Supplementary legends**

662

663 **Table S1.** *Haemonchus contortus* worm burden averages (minimum and maximum values) of
664 Ile de France and Santa Ines suckling lambs experimentally infected.

665

666 **Figure S1.** Eggs per gram of faeces (EPG) of the eight Santa Ines lambs experimentally
667 infected with *Haemonchus contortus*.

668

669 **Figure S2.** Eggs per gram of faeces (EPG) of the eight Ile de France lambs experimentally
670 infected with *Haemonchus contortus*.

671

672 **Table S2.** Mucin stain classification of abomasal tissue (fundus and pylorus) sections of
673 Santa Ines and Ile de France suckling lambs experimentally infected with *Haemonchus*
674 *contortus* and non-infected control.

675

676 **Table S3.** Live weight mean (± standard error) in kilogram (kg) of Ile de France and Santa
677 Ines suckling lambs non-infected and experimentally infected with *Haemonchus contortus*, at
678 the day of birth, at the beginning of the infection protocol (14 days old; day of the first
679 infection) and at the end of the trial (68 days old; 54 days post first infection).

680

681 **Table 1.** Averages (±standard error) of eosinophils, mast cells and globule leukocytes in the fundus and pylorus of the abomasums of Ile de France and

682 Santa Ines suckling lambs experimentally infected with *Haemonchus contortus* and non-infected control.

Variable	Abomasal Region	Ile de France		Santa Ines		Effects (P-value)		
		Infected (n=8)	Control (n=4)	Infected (n=8)	Control (n=6)	Breed	Infection status	Breed x Infection status
Eosinophils per column of	Fundic	5.31 (±0.31)b	0.13 (±0.13)c	16.87 (±3.59)a	0.5 (±0.26)c	0.002	<0.001	0.045
mucosa	Pyloric	5.94 (±0.75)a	1.5 (±0.61)b	11.6 (±2.59)a	0.58 (±0.24)b	0.923	<0.001	0.042
Mast cells per column of mucosa	Fundic	2.68 (±0.41)b	0.5 (±0.2)c	11.81 (±2.56)a	0.42 (±0.08)c	0.003	<0.001	0.0012
	Pyloric	2.69 (±0.35)b	0.63 (±0.13)c	9.31 (±2.27)a	0.75 (±0.11)c	0.011	<0.001	0.029
Globule leukocytes per column	Fundic	0.81 (±0.16)b	0b	8.38 (±3.0)a	0b	0.012	<0.001	0.012
of mucosa	Pyloric	0.31 (±0.19)b	0b	5.06 (±2.34)a	0b	0.016	0.002	0.016
Eosinophils in the muscularis	Fundic	0.83 (±0.55)	0	2.5 (±1.37)	0	0.526	0.068	0.526
mucosae*	Pyloric	0	0	2.92 (±1.33)	0	0.065	0.065	0.065
Mast cells in the muscularis	Fundic	0.42 (±0.42)	0	1.67 (±1.26)	0	0.547	0.187	0.547
mucosae*	Pyloric	0.42 (±0.42)	0	0.75 (±0.49)	0	0.672	0.177	0.672
Eosinophils in the sub-mucosa#	Fundic	16.67 (±3.1)b	0c	67.1 (±16.66)a	0c	0.016	<0.001	0.016
	Pyloric	3.75 (±1.33)b	0b	35 (±11.63)a	0b	0.049	<0.001	0.049
Mast cells in the sub-mucosa#	Fundic	19.17 (±1.22)b	4.17 (±0.83)d	38.33 (±4.88)a	9.44 (±1.59)c	<0.001	<0.001	0.908
	Pyloric	12.08 (±1.4)b	4.17 (±1.59)c	37.5 (±4.07)a	6.11 (±4.07)b	0.012	<0.001	0.139

683 In each row, arithmetic means with different lower case letters are significantly different by Tukey test ($P<0.05$).
684 * Cells were counted in thirty randomly selected fields of view at x1000 magnification in an area of 100 μm^2.
685 # Cells were counted in thirty randomly selected fields of view at x1000 magnification in an area of 2500 μm^2.
686

687 **Table 2.** Averages (±standard error) of variables analyzed in the fundus and pylorus of the
688 abomasums of Santa Ines suckling lambs experimentally infected with *Haemonchus*
689 *contortus*.

Variable	Abomasal Region	Infected Santa Ines		
		More resistant (n=3)	Less resistant (n=5)	(P-value)
Mucosal thickness (µm)	Fundic	615 (±5.78)	571 (±15.6)	0.084
	Pyloric	643.3 (±7.64)	549 (±22.33)	0.019
Eosinophils per column of mucosa	Fundic	29 (±2.65)	10.2 (±1.24)	0.001
	Pyloric	19 (±2.65)	7.4 (±2.11)	0.034
Mast cells per column of mucosa	Fundic	20.67 (±1.45)	7.2 (±0.97)	0.001
	Pyloric	17 (±0.58)	4.8 (±0.86)	0.002
Globule leukocytes per column of mucosa	Fundic	18.7 (±1.33)	2.4 (±0.4)	0.001
	Pyloric	11.3 (±4.41)	1.4 (±0.25)	0.006
Eosinophils in the muscularis mucosae*	Fundic	6.67 (±2.02)	0	<0.001
	Pyloric	6.67 (±2.02)	0.6 (±0.6)	0.009
Mast cells in the muscularis mucosae*	Fundic	3.33 (±3.33)	0.6 (±0.6)	0.499
	Pyloric	2 (±1)	0	0.034
Eosinophils in the sub-mucosa#	Fundic	121.3 (±5.67)	34.6 (±8.18)	0.006
	Pyloric	71 (±7.57)	13.4 (±6.9)	0.036
Mast cells in the sub-mucosa#	Fundic	54.33 (±2.96)	28.8 (±1.2)	<0.001
	Pyloric	49.67 (±3.33)	30 (±2.41)	0.005

690 * Cells were counted in thirty randomly selected fields of view at x1000 magnification in an area of 100 μm^2.
691 # Cells were counted in thirty randomly selected fields of view at x1000 magnification in an area of 2500 μm^2.
692