Veterinary and Animal Science 19 (2023) 100290

Contents lists available at ScienceDirect

Veterinary and Animal Science

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

Antibody response and abomasal histopathology of lambs with haemonchosis during supplementation with medicinal plants and organic selenium

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ARTICLE INFO

Keywords:
Antibody responses
Haemonchus contortus
Histopathology
Medicinal plants
Resistance
Selenium

ABSTRACT

We evaluated the effects of dietary supplementation with medicinal plants (Herbmix) or organic selenium (Selplex) on the immune responses and histopathology of lambs infected with *Haemonchus contortus*. Twenty-seven lambs were infected and reinfected with approximately 11,000 third-stage larvae of *H. contortus* during the experiment (on days 0, 49 and 77). Lambs were divided into two supplemented experimental groups (Herbmix and Selplex) and unsupplemented group (Control). The abomasal worm counts at necropsy on day 119 were lower for Herbmix (4230) and Selplex group (3220) than to the Control (6613) which resulted in 51.3% and 36.0% of reduction, respectively. The mean length of adult female worms was in the order Control > Herbmix > Selplex (2.1, 2.08, and 2.01 cm, respectively). The specific IgG response against adults was significantly affected by time ($P < 0.001$). Serum-specific and total mucus levels of IgA in the Herbmix group were highest on day 15. Mean levels of serum IgM against adults were influenced by treatment ($P = 0.048$) and time ($P < 0.001$). The Herbmix group had strong local inflammation in the abomasal tissue, with the formation of lymphoid aggregates and the infiltration of immune cells, but the tissues of the Selplex group had higher numbers of eosinophils, globule leukocytes, and plasma cells. The lymph nodes of each animal had reactive follicular hyperplasia due to the infection. Dietary nutritional supplementation with a mixture of medicinal plants or organic selenium could improve local immune responses and thus enhance the resistance of animals to this parasitic infection.

1. Introduction

Livestock production is one of the fastest-growing agricultural sectors, currently occupying 30% of the terrestrial surface area. This growth is driven by increasing demand for livestock products due to population growth (Britt et al., 2018; Herrero et al., 2013). Grazing ruminants, however, are at constant risk of being infected by larvae of parasitic gastrointestinal nematodes (GINs). GIN infections represent the main health problem affecting ruminants and are responsible for financial losses in the livestock industry worldwide (Valcárcel et al., 2015). *Haemonchus contortus*, a blood-feeding and highly pathogenic nematode is considered the most important GIN in small ruminants. This parasite is responsible for economic losses due to losses in productivity, high mortality, and cost of treatment (Arsenopoulos et al., 2021).

The control of GINs relies on the repeated use of anthelmintics. Their excessive use, however, has led to the development of anthelmintic resistance in parasite populations (Bihaqi et al., 2020; Jackson et al., 2012; Kaplan, 2020). Interest in new alternative control strategies is growing due to the emergence of anthelmintic resistance and the demands of consumers for animal products free of chemicals. One of the solutions to reduce GIN infections can be the improvement of host immune responses by nutritional supplementation. Chronic infections, such as those caused by GINs, can prevent animals from reaching proper immunological responses due to malnutrition (McClure, 2008). Improved host nutrition, therefore, plays an important role in the sustainable control of parasitic infections in animals (Houdijk et al., 2012).

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https://doi.org/10.1016/j.vas.2023.100290
Received 27 November 2022; Received in revised form 4 February 2023; Accepted 7 February 2023
Available online 10 February 2023

The use of plants rich in bioactive compounds such as polyphenols has been considered an option for controlling GINs. The supplementation of animal diets with medicinal plants can provide nutrients necessary for tissue maintenance, homeostasis, and immune responses, thereby indirectly increasing host resistance to parasites (Hoste et al., 2016). Minerals also play essential roles in the development of proper immune responses. Trace minerals such as selenium (Se) are dietary supplements that can potentially affect immunity to parasites. Se is an essential dietary micronutrient important for initiating and enhancing innate and adaptive immune responses and is also involved in immunoregulation and preventing excessive immune responses and chronic inflammation (Hoffmann & Berry, 2008; Huang et al., 2012).

Larvae undergo invasive and developmental processes in the abomasum during the GIN life cycle to reach the adult stage. The activity of GINs in the gastrointestinal tract causes histopathological changes in tissues, such as disruption of the abomasal mucosa, damage to epithelial cells and glands, and hyperplasia of numerous cell lineages. Damage caused by parasites also stimulates the infiltration of immune cells into abomasal tissue (Bowdridge et al., 2015). Protective host immune responses against GINs are mediated by type 2 immune responses involving the infiltration of eosinophils, globule leukocytes, and plasma cells in the abomasal tissue and an increase in the production of cytokines and parasite-specific antibodies (McRae et al., 2015). These host immune responses play important roles in regulating the damage caused by these parasites and are associated with lower egg production and excretion by adult worms, lower parasitic burden, and shorter female parasites (Machín et al., 2021).

Our previous studies found that supplementing lamb diets with dry medicinal plants increased resistance to parasitic infection (Mravčáková et al., 2019, 2021). We, therefore, hypothesised that supplementation with feed additives, such as medicinal plants or trace elements, in the diet of lambs infected with GINs would improve their immune responses. The objective of this study was therefore to evaluate the effect of diets containing a mixture of medicinal plants or organic Se on immune responses and the histopathology of the abomasa of lambs experimentally infected with the GIN *H. contortus*.

2. Material and methods

2.1. Ethics statement

The experimental protocol was conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences on 14 October 2019 (protocol code 2019/17). Permission to collect samples and carry out the experiments was granted by the participating sheep farmer.

2.2. Animals and experimental design

The experiment was part of a larger study that evaluated the effect of dry medicinal plants and organic Se on lambs infected with *H. contortus* previously described in detail by Komáromyová et al. (2021). Briefly, twenty-seven female lambs (Improved Valachian, susceptible to GIN) 3–4 months old with initial body weights of 18.52 ± 2.18 kg were housed in common stalls on a commercial sheep farm (PD Ružín – Ružín farm, Kysak, Slovakia) and maintained in its production system with free access to water during the experiment. After a 14-d period of adaptation, all parasite-free lambs were orally infected with approximately 11,000 third-stage larvae (L3) of the MHCo1 (MOSI) strain of *H. contortus* susceptible to anthelmintics. The animals were infected with 5000 L3 on D0 and re-infected with 3000 L3 on D49 and D77. Each animal was fed the Mikrop ČOJ commercial concentrate (MIKROP, Čebín, Czech Republic; 300 g dry matter (DM)/d) and meadow hay (*ad libitum*). The infected lambs were then randomly divided into three groups of 11 animals each based on their live weights (11 lambs/group, one stall/group): unsupplemented lambs (Control), lambs supplemented with dry medicinal plants (Herbmix, 100 g DM/d/lamb), and lambs supplemented with organic Se (Selplex, selenised yeast, SEL-PLEX 2300; Alltech, Nitra, Slovakia; 0.24 mg Se/animal). The Herbmix was obtained from commercial sources (AGROKARPATY, Plavnica, Slovak Republic, and Byliny Mikeš s.r.o., Čífčenice, Czech Republic) and consisted of 11.8% each of *Althaea officinalis, Petasites hybridus, Inula helenium, Plantago lanceolata, Rosmarinus officinalis, Solidago virgaurea, Fumaria officinalis*, and *Hyssopus officinalis* and 5.6% *Foeniculum vulgare*. The Herbmix contained flavonoids (9.965 g/kg), diterpenes (4.886 g/kg), and phenolic acids (3.549 g/kg) and high concentrations of quercetin-O-Hex-dHex, verbascoside, 3,5-dicaffeoyl-quinic acid, rosmarinic acid, and carnosol (Váradyová et al., 2018). Herbal and Se supplementation began on the day (D) 0 and were mixed daily with the commercial concentrate during the experimental period (119 days). Animals were killed at the end of the experiment following the rules of the European Commission (Council Regulation 1099/2009) for slaughtering procedures. To establish efficacy of the treatment of the Herbmix and Selplex group seven animals were slaughtered in each group. Two animals from each group were killed on D15 and D22 to obtain mucosal samples from the abomasum. The helminthological dissections were performed, and mucus and tissue samples from the abomasum were obtained for further analysis.

2.3. Morphometry of H. contortus

The abomasum of each animal was removed after necropsy, opened along the greater curvature, and the content was emptied into a bucket. The content was adjusted with water to a total volume of 2 L and mixed. An aliquot of 100 mL was transferred to a beaker and preserved in 10% formalin. A subsample of 10 mL was transferred to a Petri dish, and five adult males and five adult females of *H. contortus* were subsequently collected from each sample for measurement. The differentiation of adults was based on morphological differences in the reproductive system. Parasites or parts of parasites were photographed using a Leica DFC 290 camera under a Leica DM 4000 B microscope (Leica Microsystems, Wetzlar, Germany). The morphometry of the adults was analysed using Leica Application Suite 4.13 software (Leica Microsystems, Wetzlar, Germany).

2.4. Blood analysis

Samples of blood were collected from the jugular vein of each animal on D0, D15, D35, D49, D63, D77, D98, and D112 using a 21-gauge needle and syringe. The samples were placed in microtubes containing 1.6 mg/mL EDTA-K3 (Sarstedt AG & Co, Nümbrecht, Germany). The numbers of immune cells (total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were immediately determined using an Abbott CELL-DYN 3700 automated haematological analyser (Global Medical Instrumentation, Inc., Ramsey, USA). Blood samples for sera were collected in 10 mL serum-separator tubes (Sarstedt AG & Co, Nümbrecht, Germany) and centrifuged at 1200 *g* for 10 min at room temperature. The sera were stored at −80 °C until analysis.

2.5. Mucus

Mucosal samples were obtained from the abomasa on D15, D22, and D119 for determining the levels of total local IgA. The mucus was collected after necropsy by gently scraping the mucosal surface of the abomasum using a glass microscope slide. The mucus was diluted in a buffer (pH 7.1; Na2HPO4 0.1 M; NaCl 0.05 M; NaN3 3 mM; PMSF 1 mM; EDTA 5 mM) at a rate of 2.5 mL per gramme of mucus, homogenised, and centrifuged at 18 000 *g* at 4 °C for 30 min Amarante et al. (2005). The supernatant was then collected and stored at −20 °C until analysis.

2.6. Preparation of H. contortus antigens

H. contortus somatic antigens were prepared similarly to a previously described assay (Mravčáková et al., 2019). Briefly, antigens were obtained from 50 000 L3 or adult worms harvested from the abomasa of sheep experimentally infected with approximately 5000 L3. The larvae and adults were washed twice in phosphate-buffered saline (PBS, pH 7.4), diluted to a volume of approximately 15 mL, and homogenised in an ice-cold glass tube using an HD3100 Sonopuls ultrasonic homogeniser (BANDELIN electronic, Berlin, Germany). The homogenate was centrifuged (Heraeus Megafuge 16R, Thermo Fisher Scientific, Waltham, USA) at 4500 *g* at 4 °C for 5 min. The supernatant was concentrated in 3000 MWCO VIVASPIN tubes (Sartorius, Goettingen, Germany) at 4000 *g* at 4 °C for 100 and 40 min for larvae and adults, respectively. The protein concentrations of the larval and adult somatic antigens were measured using the Bradford protein assay (Bio-Rad Laboratories, München, Germany).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The levels of specific anti-*H. contortus* IgG, IgA, and IgM in the serum and total IgA in the mucus were determined using an indirect enzyme-linked immunosorbent assay (ELISA). ELISAs were performed using larval and adult somatic *H. contortus* antigens to detect the parasite-specific antibodies, and rabbit anti-sheep IgA antibody (Bethyl Laboratories, Inc. Montgomery, USA) to detect the total IgA, similar to Mravčáková et al. (2019). The larval and adult somatic antigens were diluted to a final concentration of 5 μg/mL for specific IgG, IgA, and IgM in the serum, and anti-sheep IgA antibody for total mucus IgA was diluted 1:500 in carbonate buffer (pH 9.6) and bound to Nunc microtitre plates (Thermo Fisher Scientific, Roskilde, Denmark) at 4 °C overnight followed by three washes with 0.5% Tween 20 in PBS (pH 7.4, PBS-T). Non-specific bonds were blocked with 0.5% non-fat dry milk in PBS by incubating the plates for 30 min at room temperature, and the wells were then washed again three times with PBS-T, as above. The serum samples were diluted 1:100 for IgG and IgM and 1:25 for IgA, and mucosal samples were diluted 1:10 and incubated at 37 °C for 1 h. The wells were again washed as above, and bound antibodies were detected by incubating at 37 °C for 1 h with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Sigma-Aldrich, Hamburg, Germany), rabbit anti-sheep IgM (Abcam, Cambridge, USA), and rabbit anti-sheep IgA (Bio-Rad Laboratories, Inc., Kidlington, UK) diluted 1:5000, 1:50 000, and 1:10 000, respectively. The wells were again washed with PBS-T and 0.05 mol/L of the substrate o-phenylenediamine (Sigma-Aldrich, Hamburg, Germany) in citrate buffer (pH 4.7) and with 0.005% H_2O_2 to induce a colour reaction. The reaction was stopped using 2 M H_2SO_4 after incubation for 15–30 min at room temperature in the dark. The results are expressed as optical densities measured at 492 nm using an Apollo 11 LB913 Elisa absorbence reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

2.8. Histopathology

Histological examinations were performed similar to those described by Petrič et al. (2021). Tissue samples from the fundus part of the abomasum and mesenteric lymph nodes were removed, washed in a phosphate buffer solution (0.1 M, pH 7.4), transferred to plastic containers, and routinely fixed in 10% neutral buffer formalin. The fixed tissues were processed with a series of reagents, embedded in Paraplast PLUS paraffin blocks (Leica, Buffalo Grove, USA), and subsequently cut using a rotary microtome into Sections 3.5 μm thick. The paraffin slides were automatically stained with haematoxylin and eosin (HE; Varistain Gemini, Thermo Scientific, Runcorn, UK) for evaluating eosinophils and globule leukocytes. Mucus was stained with Periodic Acid-Schiff according to Hotchkiss – McManus stain. The histological samples were evaluated using an Axio Lab. A1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam ERc5s digital camera (Carl Zeiss, Jena, Germany). Cells in the abomasal tissue were counted in four randomly selected fields in a 0.01 mm^2 area. The results are presented as the total number of cells per section. The intensity of inflammation was scored on a three-point scale, where + represents no or minimal inflammation, ++ represents moderate inflammation, and +++ represents severe inflammation. The total number of lymphatic follicles was counted, and the surface area was measured, for each lymph node. Photographs were analysed and recorded using ZEN 2.3 software (Blue edition; Carl Zeiss Microscopy GmbH, 2011).

2.9. Calculations and statistical analysis

Analyses of variance (ANOVAs) (GraphPad Prism, GraphPad Software, Inc., San Diego, USA) were used for analysing the peripheral blood cells parameters and humoral responses in serum as repeated-measures mixed models representing the three animal groups (Control, Herbmix, and Selplex) and sampling days (D0–D119). Effects included in the model were treatment, time, and interaction. Differences between the Control group and both treated (Herbmix and Selplex) groups were analysed using a two-way ANOVA with a Bonferroni *post hoc* test. Percent changes in abomasal tissues were calculated as the number of animals with corresponding histopathological changes compared to all animals in the group (Table 3). Data for morphometry and humoral responses in the abomasal mucus were evaluated by multiple comparisons using one-way ANOVAs. Results were considered significant at $P < 0.05$.

3. Results

3.1. Parasitological data

Results have been previously described in detail by Komáromyová et al. (2021). Egg output (eggs per gram, EPG) during the experimental period from D28 for Herbmix, Selplex and Control group were in ranges of 5188 – 16,675, 4733 – 10,075 and 7525 – 19,867, respectively. Treatment after D105 with Herbmix or organic Se produced significant reduction of faecal egg count ($P < 0.01$) with reduction of 53.9 ± 0.8% and 52.3 ± 0.8%, respectively. The abomasal worm counts at necropsy on D119 were lower for Herbmix (4230) and Selplex group (3220) than to the Control (6613) which resulted in 51.3% and 36.0% of reduction, respectively.

3.2. Morphometry of H. contortus

The body lengths of *H. contortus* females ranged from 15.19 to 28.58 mm, and the lengths of *H. contortus* males ranged from 10.72 to 15.79 mm. The lengths of both sexes did not differ significantly ($P > 0.05$) between the experimental groups at necropsy on D119 (Table 1), but the parasites in the Selplex group were shorter than the parasites in the Herbmix and Control groups.

Table 1
The body lengths of female and male worms in the Control, Herbmix, and Selplex groups at the end of the experiment ($n = 7$).

Worm body length (mm)	Control	Herbmix	Selplex	SD	P
Female body length	21.46	20.83	20.14	2.599	0.299
minimum	15.19	16.26	16.30		
maximum	25.23	25.4	28.58		
Male body length	13.92	13.73	13.12	1.261	0.780
minimum	11.77	11.58	10.72		
maximum	17.39	15.79	16.4		

SD, standard deviation.

3.3. Peripheral blood-cell counts

Total leukocytes, neutrophils, and monocytes were significantly influenced by treatment ($P < 0.004$, $P < 0.037$, and $P < 0.006$, respectively) and time affected all the cells (Table 2). None of the counts of immune cells were significantly affected by the interaction between treatment and time ($P > 0.05$).

3.4. Antibody responses

Mean serum IgG antibody responses against *H. contortus* L3 and adults were similar and did not differ significantly ($P = 0.122$ and $P = 0.580$, respectively) between experimental groups during the experiment (Fig. 1). IgG against L3 slightly increased in the Selplex group after the first re-infection and remained higher than in the other groups until the end of the experiment. The IgG response against adults was significantly affected by time ($P < 0.001$). The infection caused an elevation of serum IgA antibodies against L3 antigen in the Herbmix group on D15, which subsequently decreased until the first re-infection. The IgA levels increased similarly in each group after both re-infections but did not differ significantly during the experiment (Fig. 2A). The serum IgA antibody response against adult antigen increased after the first re-infection on D49, peaking at D63 in the Herbmix and Selplex groups and at D77 in the Control group (Fig. 2B). Mean serum IgA levels against adults were influenced by time ($P < 0.0001$) and the treatment × time interaction ($P = 0.0428$) and differed significantly between the Control and Herbmix groups ($P < 0.05$) and between the Control and Selplex groups ($P < 0.01$) on D77. The serum IgM antibody response against L3 was affected by time ($P < 0.001$) and gradually increased throughout the experiment (Fig. 3A). All treatments had similar IgM antibody responses against the adult antigens, with the peak at D77. Mean serum IgM levels against adults were influenced by treatment ($P = 0.048$) and time ($P < 0.001$).

The levels of total IgA in the mucus of the Control, Herbmix, and Selplex groups did not differ significantly ($P = 0.296$) on different experimental days. The level of mucus IgA on D15 and D22, however, was higher in the Herbmix than the other groups (Fig. 4).

Table 2
Total leukocyte and differential counts of immune cells in the Control, Herbmix, and Selplex groups during the experiment ($n = 7$).

Item	Day	Control	Herbmix	Selplex	SD	Significance of effect		
						Treatment	Time	Treatment × time
Total leukocytes (g/L)	0	8.97	10.6	10.4	2.58	0.004	< 0.001	0.953
	15	9.03	9.04	9.97	1.73			
	35	7.41	8.19	9.29	1.41			
	49	7.04	6.58	7.93	1.07			
	63	7.81	7.46	8.52	1.20			
	77	6.93	7.08	8.61	1.27			
	98	6.75	7.00	8.86	1.97			
	112	6.40	7.13	7.38	1.17			
Neutrophils (g/L)	0	3.08	4.36	3.50	1.73	0.037	< 0.001	0.928
	15	3.05	3.14	3.27	0.851			
	35	1.95	2.59	2.43	0.758			
	49	2.17	1.86	2.43	0.708			
	63	2.08	2.30	2.36	0.707			
	77	1.88	2.42	2.60	0.652			
	98	1.65	2.35	2.80	1.95			
	112	1.96	2.50	2.24	0.888			
Lymphocytes (g/L)	0	2.20	1.86	2.18	1.01	0.157	0.002	0.969
	15	1.55	1.44	1.70	0.588			
	35	2.18	2.65	3.12	1.18			
	49	2.29	2.17	2.88	0.770			
	63	2.75	2.58	2.52	0.982			
	77	2.72	2.24	2.68	1.07			
	98	2.23	2.01	2.19	0.634			
	112	2.16	2.11	2.36	0.837			
Monocytes (g/L)	0	2.95	2.86	3.45	0.819	0.006	< 0.001	0.981
	15	3.47	3.20	3.70	1.03			
	35	2.64	1.88	2.71	0.936			
	49	1.99	2.02	2.12	0.795			
	63	1.92	1.64	2.59	1.15			
	77	1.42	1.55	2.31	1.13			
	98	1.34	1.30	2.29	0.671			
	112	1.59	1.53	2.10	0.769			
Eosinophils (g/L)	0	0.335	0.089	0.254	0.213	0.215	< 0.001	0.443
	15	0.364	0.288	0.437	0.235			
	35	0.132	0.093	0.126	0.123			
	49	0.138	0.110	0.087	0.097			
	63	0.161	0.131	0.220	0.139			
	77	0.173	0.171	0.239	0.154			
	98	0.189	0.102	0.123	0.117			
	112	0.072	0.224	0.127	0.108			
Basophils (g/L)	0	0.420	1.45	0.708	0.838	0.140	0.006	0.849
	15	0.586	0.976	0.855	0.511			
	35	0.520	0.978	0.928	0.570			
	49	0.460	0.414	0.406	0.288			
	63	0.903	0.815	0.828	0.787			
	77	0.450	0.762	0.763	0.470			
	98	1.35	1.24	1.46	0.901			
	112	0.612	0.775	0.561	0.495			

SD, standard deviation.

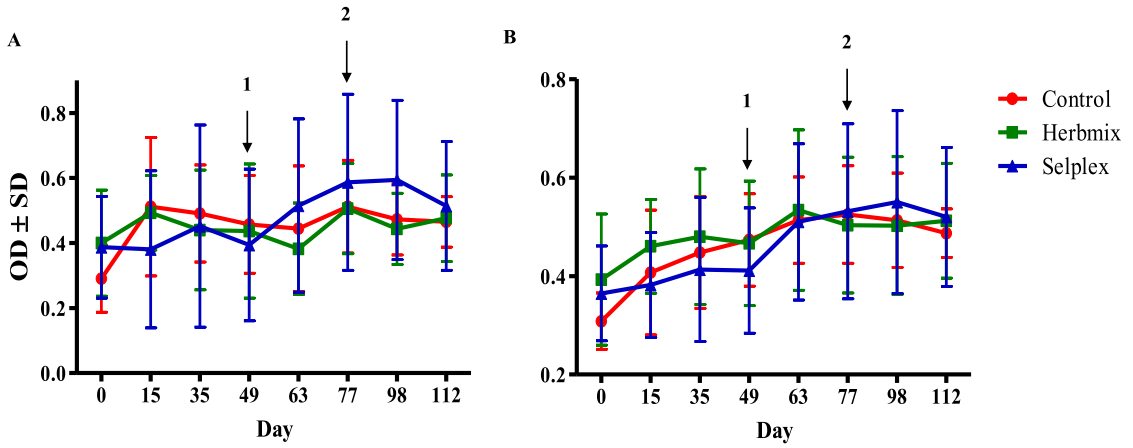

Fig. 1. Serum-specific IgG responses against *Haemonchus contortus* larval (A) and adult (B) antigens in lambs in the Control, Herbmix, and Selplex groups during the experiment. 1, First re-infection; 2, second re-infection.

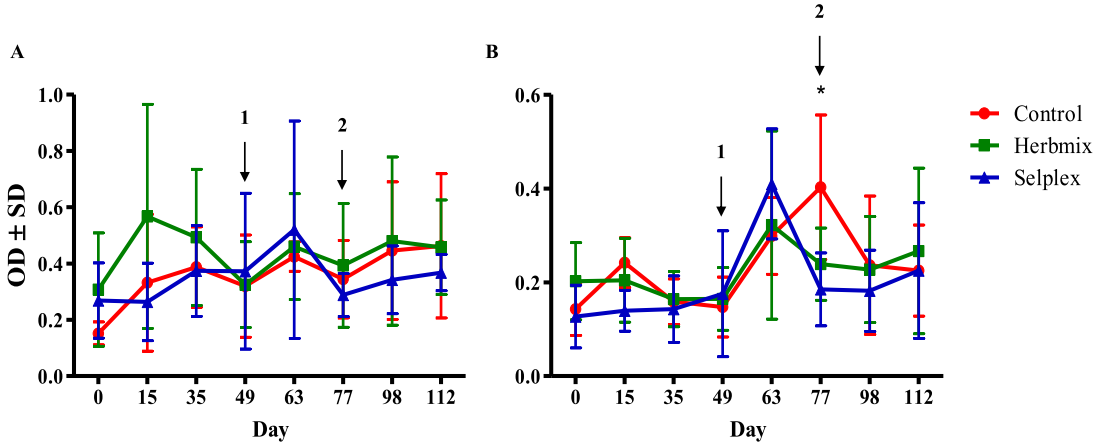

Fig. 2. Serum-specific IgA response against *Haemonchus contortus* larval (A) and adult (B) antigens in lambs in the Control, Herbmix, and Selplex groups during the experiment. 1, First re-infection; 2, second re-infection.

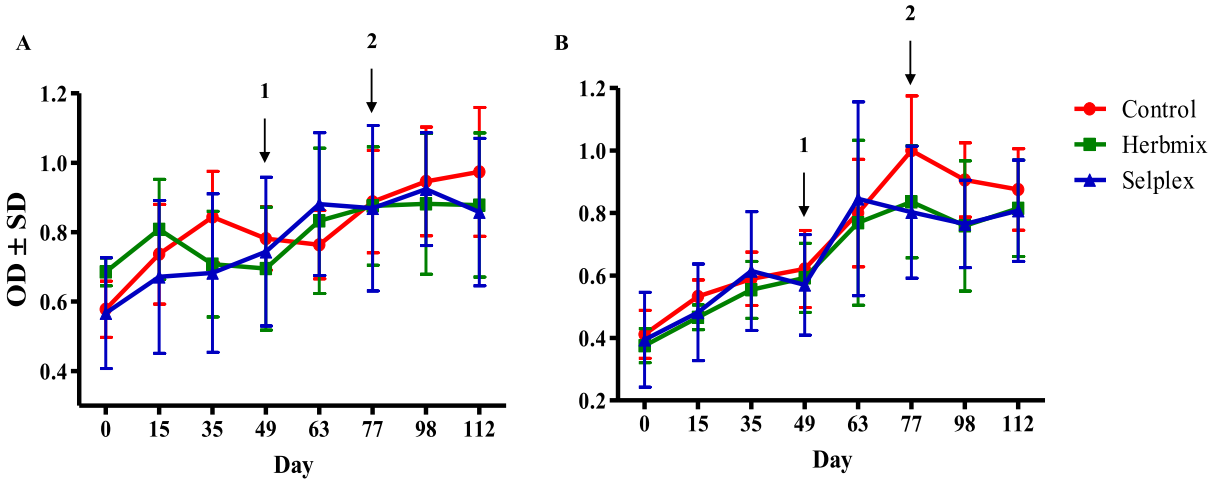

Fig. 3. Serum-specific IgM response against *Haemonchus contortus* larval (A) and adult (B) antigens in lambs in the Control, Herbmix, and Selplex groups during the experiment. 1, First re-infection; 2, second re-infection.

3.5. Histopathology

The changes in the abomasal tissues of the lambs infected with *H. contortus* were accompanied by varying degrees of inflammation. Inflammatory cells and foci of infiltration were in the vicinity of the lamina propria, and a few inflammatory cells were scattered in the connective tissue of the mucosa (Fig. 5A). Organ architecture in the Control and Selplex groups was preserved except for the loss of structure

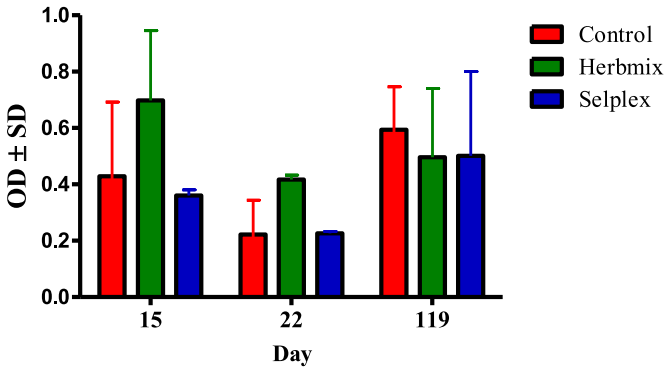

Fig. 4. Total mucosal IgA in lambs in the Control, Herbmix, and Selplex groups on days 15, 22, and 119 post-infection.

at the more intense inflammatory infiltrate site. Inflammation in the Herbmix group was moderate to severe (Fig. 5B), and the tissue architecture was not preserved. Some animals in the Selplex group had moderate mucosal damage, with shallowing of the gastric pits and segmental mucosal hypersecretion (Fig. 5C). In one sample from the Selplex group, a degenerated larva was surrounded by strong inflammation with few eosinophils (Fig. 5D). Other changes in the abomasal tissue included hypertrophy of the mucosa, damage to epithelial cells, hyperplasia of mucus-producing cells (Fig. 5E), dilatation of glands, and damaged glands (Fig. 5F), which were similar in each group (Table 3). Each animal in the Herbmix group had lymphoid aggregates in the tissues. Some animals from both treated groups had concurrent signs of glandular dilatation and damage. Eosinophils were mostly near the lamina propria in the Control group but were also scattered throughout the mucosa in the treated groups. The number of eosinophils in the abomasal mucosa was lowest in the Control group (Fig. 6A). Globule leukocytes were mainly scattered in the mucosa and abomasal glands (Fig. 5G). Similarly, the treated animals had a higher concentration of these immune cells in the abomasal tissue (Fig. 6B). Plasma cells were mainly scattered throughout the mucosa, with the highest numbers in the Selplex group (Fig. 6C). Lymphocytes in the inflammatory infiltrate accounted for 60% of all immune cells but accounted for up to 100% in a few animals in the Control and Herbmix groups (Fig. 6D). The intensity of inflammation was highest in the Herbmix group (Fig. 6E), where inflammation of the abomasum was moderate to strong in each animal.

The number and average size of the lymphatic follicles in the Control, Herbmix, and Selplex lambs at the end of the experiment are presented in Table 4. The number of lymphatic follicles was higher in the Selplex group than the Control and Herbmix groups. The follicles varied in size and mostly contained germinal centers. The lymph nodes of each animal had reactive follicular hyperplasia (Fig. 7A). A few animals in the Herbmix and Selplex groups had enlarged paracortical areas and medullary cords, with the number of lymphocytes higher in the Herbmix group and the number of lymphocytes and plasma cells higher in the Selplex group. Eosinophil counts were variable in the medullary and peritrabecular sinuses. One animal in the Herbmix group had lymphocytic thickening in the medulla, with strong inflammation and eosinophils in the centre of the necrosis (Fig. 7B). Another animal in the same group had thickened vessel walls and vessel obliteration in the cortical zone of the lymph node.

4. Discussion

The study was focused on the effect of feed supplements on the immune responses and histopathology of infected lambs. Host nutritional status affects the ability to develop strong immune responses against gastrointestinal infections (Hoste et al., 2016), which subsequently influence the establishment and development of parasites. Counting worms and measuring their length can therefore be used to assess the strength of immune responses (Stear et al., 1999). The typical length of *H. contortus* in females ranges between 18 and 30 mm, whereas males usually range between 10 and 20 mm (Gareh et al., 2021). The mean lengths of females and males in each experimental group in our study were within these ranges. The parasites, however, were shorter in both treated groups than the Control group and were shortest in the Selplex group. This difference could be attributed to the increased number of immune cells, such as eosinophils and globule leukocytes, in the abomasum in this group. Lacroux et al. (2006) observed a strong negative correlation between female length and the populations of eosinophils, mast cells, and globule leukocytes in the abomasum. Other studies, however, reported that IgA, presumably together with eosinophils, played a role in the control of this infection by influencing parasitological parameters, such as worm length, fecundity, and worm burden (Hernández et al., 2016; Ortega et al., 2022; Strain & Stear, 2001). The specific or mucosal IgA response was similar in each experimental group towards the end of the experiment, so the difference in worm length could only be associated with the infiltration of immune cells in the abomasal tissue.

Immunity against GINs involves both cellular and humoral responses, which are closely interdependent and interact. Innate immunity represents the first line of defence of the host, which is important for the initial response to pathogens and for initiating and regulating acquired immunity (McRae et al., 2015). Innate immunity consists of various components, where the main cellular components are immune cells, such as monocytes, neutrophils, basophils, and eosinophils (Romo et al., 2016). Total leukocyte counts in our study were significantly affected by treatment and were higher in both treated groups towards the end of the experiment. The higher number of white blood cells in these groups could be attributed to a large increase in neutrophil and monocyte counts. Ortolani et al. (2013) observed that sheep with a higher level of parasitism had lower total leucocyte counts, indicating that these animals more susceptible to haemonchosis were characterised by leukopaenia. Neutrophil and eosinophil counts were also lower in these animals 57 d after infection. Similarly, lambs in the Control group in our study had the lowest leukocyte and neutrophil counts after D77, associated with higher abomasal worm counts and significantly higher egg output from D91 until the end of the experiment (Komáromyová et al., 2021). Dietary supplementation with medicinal plants or organic Se could therefore probably increase the resistance of these animals to haemonchosis by affecting immune cells in the blood responsible for protective immune responses. This assumption was supported by Bowdridge et al. (2015), who reported significantly higher levels of leukocytes and neutrophils, a higher level of monocytes in the blood, and a lower total abomasal worm burden in a parasite-resistant breed of sheep compared to a susceptible breed of sheep. Other innate immune cells in the bloodstream necessary for defence against GINs, such as eosinophils and basophils, however, were not affected by different treatments in our study.

Humoral immune responses, as a part of adaptive immunity, represent the second line of defence against GINs and are characterised by the increased production of specific antibodies by B lymphocytes, such as IgG, IgA, IgM, and IgE (Marshall et al., 2018). These immunoglobulins have been widely studied in small ruminants as biomarkers of resistance to GINs (Aboshady et al., 2020). Several studies have suggested that IgG level usually increases during infection, and higher levels are associated with the resistance of sheep to some GINs. For example, Muñoz-Guzmán et al.(2006) observed a higher level of IgG against larvae between weeks 9 and 15 after infection with *H. contortus* in a highly resistant sheep breed compared to a breed with a low resistance to haemonchosis. This increase and maintenance of serum antibodies in resistant sheep represent the development of the Th2-type immune response. Similarly, Escribano et al. (2019) and Albuquerque et al. (2019) reported higher levels of parasite-specific IgG in a breed of sheep resistant to haemonchosis.

In addition to breeding livestock for nematode resistance, nutritional

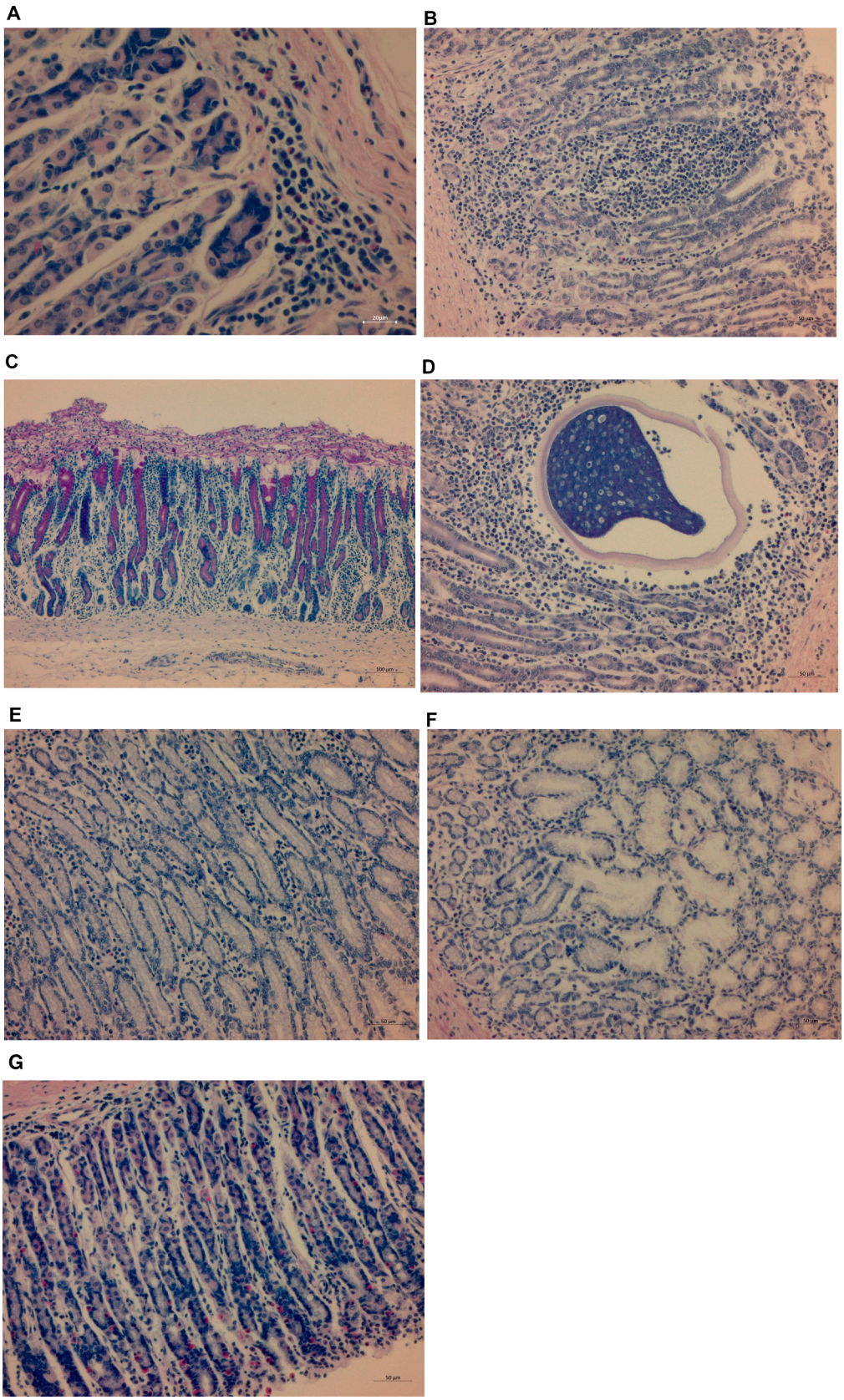

Fig. 5. Histopathological sections of abomasa from lambs infected with *Haemonchus contortus*. **A,** Sections stained with haematoxylin and eosin (HE) (200 ×) showing lymphocytes, eosinophils, and plasma cells diffusely infiltrating the lamina propria and within lymphoid aggregates. **B,** Sections stained with HE (200 ×) showing inflammatory cells and aggregates of inflammatory cells in the connective tissue of the mucosa. **C,** Section stained with P.A.S. acc. Hotchkiss – McManus (100 ×) showing the presence of mucus in the abomasal mucosa and the hypersecretion of surface mucus in the fundus region of the abomasum. Distinct cellular infiltrates accumulated throughout the mucosa. **D,** Sections stained with HE (200 ×) showing the presence of degenerated larvae with a thick capsule surrounded by strong inflammatory infiltration, with a few eosinophils in the basal region of the abomasal mucosa. **E,** Sections stained with HE (200 ×) showing hyperplasia of mucus-producing cells, with inflammatory cells scattered throughout the connective tissue of the mucosa. **F,** Sections stained with HE (200 ×) showing the dilatation of abomasal glands. **G,** Sections stained with HE (200 ×) showing abomasal glands with numerous globule leukocytes.

Table 3
Changes in the abomasal mucosa in the Control, Herbmix, and Selplex groups at the end of the experiment ($n = 7$).

Effect	Control	Herbmix	Selplex	SD	P
Hypertrophy of mucosa (%)	14.3	14.3	14.3	0.00	–
Epithelial cell damage (%)	42.9	57.1	57.1	7.31	0.848
Hyperplasia of mucus-producing cells (%)	71.4	57.1	57.1	7.11	0.840
Dilatation of glands (%)	14.3	57.1	42.9	7.11	0.272
Damage of glands (%)	42.9	42.9	42.9	0.00	–
Dilatation and damage of glands (%)	0	42.9	14.3	5.75	0.126
Presence of lymphoid aggregates (%)	71.4	100	57.1	6.23	0.177
Presence of oedema (%)	0	14.3	0	3.12	0.387

SD, standard deviation.

interventions can also potentially indirectly improve host resistance by improving immunity to parasites (Athanasiadou et al., 2008). These effects arise from an increased supply of nutrients or bioactive compounds. The IgG responses against larvae and adults in our study slightly increased during the infection but were similar in each experimental group. In our previous study, we also observed an increase in the IgG levels against *H. contortus* in infected lambs and groups treated with medicinal plants throughout the experiment. In that case, the infected groups treated with two different mixtures of medicinal plants, however, had significantly higher IgG levels 22 days post-infection than did infected unsupplemented animals, suggesting that nutritional supplementation could improve host immune responses (Mravčáková et al., 2019). Herbmix in our study, however, did not affect the IgG response, probably due to the lower concentration of bioactive compounds. In our previous study, we did not record changes in the IgG response after the supplementation of *H. contortus* infected lambs with a mixture of *Artemisia absinthium* and *Malva sylvestris* during a 75-d experiment (Mravčáková et al., 2020). We suggested that the effect of medicinal plants on the health of animals depended on the combination of bioactive compounds from different botanical families and that using only two plants with a low variety of these compounds was not adequate. Our mixture in this study contained nine medicinal plants, but the amount or concentration of their bioactive compounds or their combination was not sufficient to influence the antibody response of sheep to parasitic infection. Lambs treated with organic Se, however, had slightly higher IgG levels against larvae after the first re-infection. This level remained higher until the end of the experiment. Se is an essential trace element for proper health, production, and immunity. Supplementation with organic Se can therefore improve the rate of growth, the humoral immune response, and the antioxidant status of lambs (Kumar et al., 2009). The differences between IgG antibody responses in our study were thus not significant, so Se supplementation, similar to Herbmix supplementation, was insufficient to affect the IgG antibody response in the infected lambs. The level of IgG was consistent with the intensity of the parasitic infection (Komáromyová et al., 2021).

IgA is another immunoglobulin that is a good indicator of the resistance of lambs to *H. contortus* infection (Salgado et al., 2022). IgA is produced primarily in the gastrointestinal tract and is subsequently transported by the blood to mucosal secretions (De Cisneros et al., 2014), so levels are usually higher in abomasal mucus than serum or plasma (Shaw et al., 2012). The levels of this immunoglobulin tend to increase during GIN infections (Cériac et al., 2019; Escribano et al., 2019), and higher levels in the serum have been correlated with fewer eggs in the faeces, smaller adult females, and lower fecundity (Stear et al., 1995). These higher levels, however, are predominantly in resistant breeds of animals (Bowdridge et al., 2013). The level of specific IgA against a larval antigen in our study was highest in the Herbmix group on D15 of the experiment and total IgA was also the highest in abomasal mucus in this group, but the differences between groups were not significant. Salgado et al. (2022) did not find any correlation between serum and mucus IgA levels at the end of a 56-d experiment, but the levels were positively correlated on D15 and D28. Cruz-Tamayo et al. (2021) found differences between serum IgA levels against *H. contortus* between susceptible and resistant sheep breeds, but only 14 d post-infection. The mixture of medicinal plants in our study may have helped to induce a stronger immune reaction of the animals to infection at the beginning of the experiment, thus improving the resistance of these animals to H. contortus infection. The specific IgA levels after D49 in our study, however, were similar in each group until the end of the experiment. Similarly, in our previous study with the same mixture of medicinal plants and organic zinc supplemented for 70 d, the serum IgA levels were similar in all experimental groups (Váradyová et al., 2018). Levels of IgA against adult antigens were lower until D49 in our current study. Low levels of plasma IgA can be caused by a low intensity of infection in animals with weaker immune responses or in heavily infected animals where most mucosal IgA is bound to parasites (De Cisneros et al., 2014). IgA levels in each group in our current study increased after the first re-infection, peaking at D63 in the Selplex group and D77 in the Control group. Escribano et al. (2019) reported that IgA levels were similar in susceptible and resistant lambs, but the response was usually slower in the susceptible lambs. Similarly, Se supplementation in our study could improve resistance and help induce immune response sooner after re-infection. The high IgA level in the Control group, however, may also have been caused by the high intensity of infection, which the highest faecal egg count proves on D77 of the experiment (Komáromyová et al., 2021).

IgM represents the first class of antibodies produced during the initial exposure to antigens (Boes, 2000). Few studies, however, have investigated the responses of sheep IgM during GIN infection. Schallig et al. (1995) reported only a moderate increase in IgM levels in serum against either larval or adult antigens during a primary or challenge infection with *H. contortus* in sheep, without significant differences. IgM levels did not differ significantly between resistant and susceptible breeds of lambs infected with *H. contortus* (Shakya et al., 2011). IgM levels in our study gradually increased similarly in each experimental group during the experiment. The increase in IgM level against the adult antigen, however, was faster after the first re-infection. These results suggest that IgM does not play an important role in sheep infected with *H. contortus*.

Infection with *H. contortus* increases the production of immune cells and specific antibodies, but local immune responses are more important for protection because adult parasites reside in the abomasal mucosa (Muñoz-Guzmán et al., 2006). The role of local antibodies in the immunoprotection against GINs has already been demonstrated. For example, Snoeck et al. (2006) found that IgA was the main antibody in the mucosa preventing infective agents from disrupting the mucosal barrier. Local mucosal IgA activity has been associated with a reduction in the length and fecundity of *H. contortus* and with the early expulsion of worms (Amarante et al., 2005), whereas resistant animals have higher IgA levels (Albuquerque et al., 2019; Bowdridge et al., 2013). Total mucosal IgA levels in our experiment were not strongly affected by the different dietary treatments, but the animals supplemented with the medicinal plants had stronger mucosal IgA responses than did the other two groups at the beginning of the experiment. Mucosal and serum IgA levels have been strongly correlated (Martinez-Valladares et al., 2005), and the levels of serum IgA against a larval antigen in our study were higher on the same days in the group supplemented with Herbmix. Medicinal plants contain various bioactive compounds that have immunomodulatory and immunostimulatory activity, which could have stimulated the production of IgA antibodies (Di Sotto et al., 2020). The IgA level in all groups, however, remained similar as the infection progressed, even though supplementation with Herbmix helped lambs achieve a stronger onset of this antibody in the mucus and serum.

H. contortus larvae developing within abomasal glands can cause major damage to the abomasal tissue, accompanied by the dilatation of and damage to glands, hyperplasia in mucosal cells, and the loss of chief

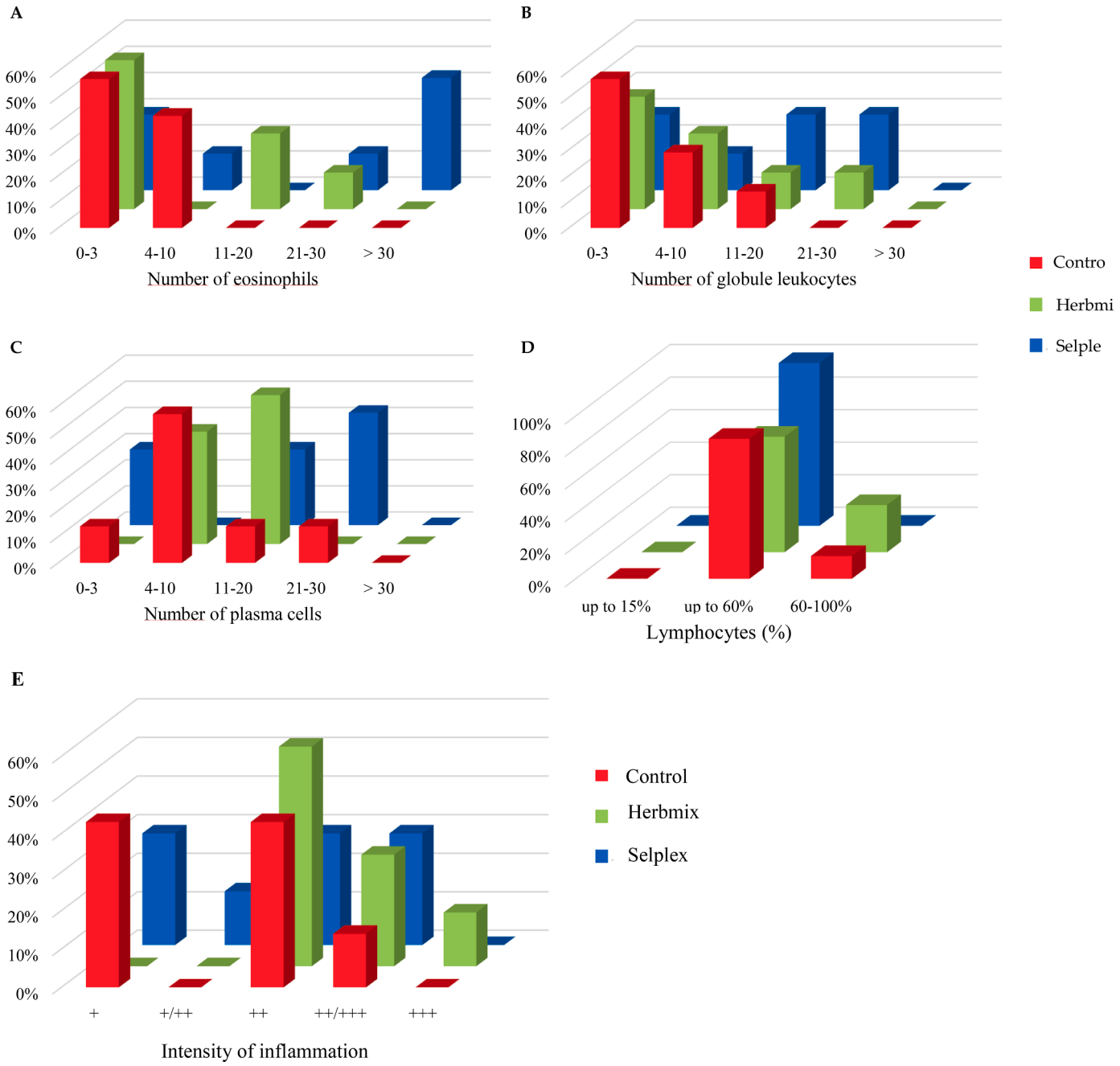

Fig. 6. Immune cells and intensity of inflammation in the abomasa of lambs infected with *Haemonchus contortus* and fed different diets. A, Number of eosinophils. B, Number of globule leukocytes. C, Number of plasma cells. D, Percentage of lymphocytes in the inflammatory infiltrate. E, Intensity of inflammation.

Table 4
Average numbers and sizes of lymphatic follicles in the Control, Herbmix, and Selplex groups at the end of the experiment ($n = 7$).

Parameter	Control	Herbmix	Selplex	SD	P
Number of lymphatic follicles	45	39	51	15.3	0.843
Size of lymphatic follicles (μm)					
minimum	264.4	270.9	216.9	47.22	0.190
maximum	693.7	639.0	771.1	153.7	0.298
Average sizes	479.1	455.0	494.0	318.6	0.974

SD, standard deviation.

and parietal cells. After reaching the adult stage, parasites pass into the abomasal lumen where they feed on blood, move, and cause general histological changes, such as superficial damage to epithelial cells, hypertrophy of the mucosa, and oedema, followed by the formation of lymphoid aggregates and the infiltration of inflammatory cells in response to tissue damage (Simpson, 2000; Stear et al., 2003). In our experiment, we observed histopathological changes in abomasal tissues typical for haemonchosis. Most of the changes, however, were similar in each experimental group. Dilatation and damage to glands, lymphoid aggregates, and oedema were more notable in the Herbmix group than the Control and Selplex groups, probably due to the stronger inflammation in this group. Our previous study also found that inflammation was strongest in the abomasa of lambs infected with *H. contortus* and supplemented with a mixture of *A. absinthium* and *M. sylvestris* compared to the unsupplemented control (Mravčáková et al., 2021). The

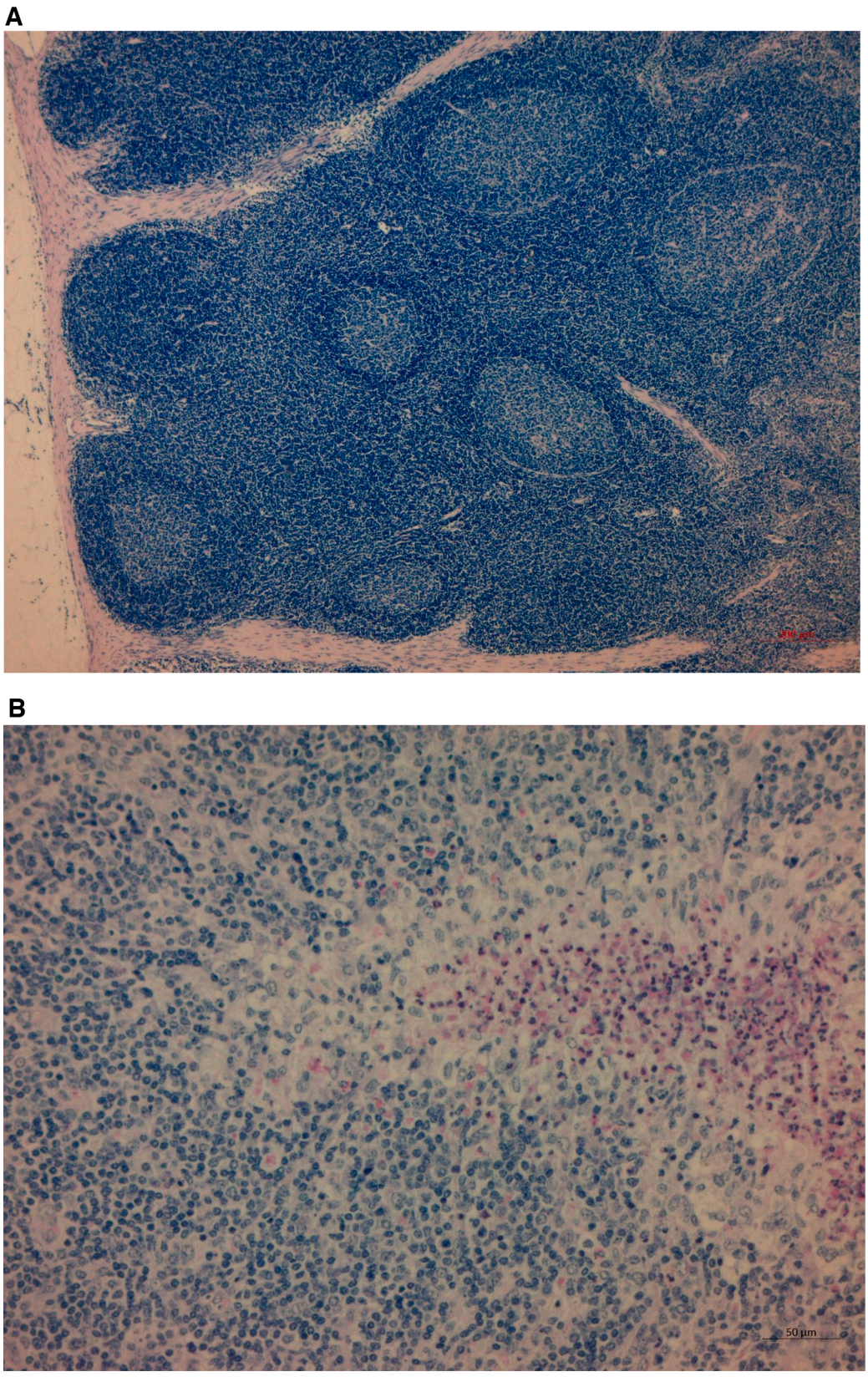

Fig. 7. Histopathological sections of mesenteric lymph nodes from lambs infected with *Haemonchus contortus*. **A,** Sections stained with haematoxylin and eosin (HE) (40 ×) showing reactive follicular hyperplasia. The follicles vary in size and contain germinal centers. **B,** Sections stained with HE (200 ×) showing necrosis and strong infiltration of eosinophils in the center of the lymph node.

prolonged and more severe inflammation in the group supplemented with medicinal plants may have been due to the delayed and incomplete defence against parasites, where a large number of parasites can cause lesions of the abomasal mucosa, which can lead to stronger local inflammation (Toscano et al., 2019). The number of adult parasites in the abomasum at the end of the experiment, however, was highest in the Control group (Komáromyová et al., 2021). The more-intense inflammation in this group, though, may have been due to the increased resistance of these animals to parasitic infection. Similarly, Lins et al. (2022) observed more-intense local inflammation in infected resistant than susceptible lambs, which led to the infiltration of eosinophils, mast cells, globule leukocytes, and lymphocytes in the mucosa involved in immune responses. Cytokine levels should also be measured in the future to better understand why supplementation with medicinal plants can cause stronger inflammation in abomasal tissue because cytokines are responsible for mediating and regulating inflammation (Zhang & An, 2007).

Immune responses against GINs are characterised by the accumulation of inflammatory cells, such as eosinophils, globule leukocytes, plasma cells, and lymphocytes, in the infected tissue (Lacroux et al., 2006; Pérez et al., 2001). The number of blood eosinophils in parasitic diseases increases greatly in response to Th2 cytokines, and blood eosinophils are subsequently recruited into the infected tissue, contributing to tissue inflammatory responses (Shin et al., 2009). Eosinophils play an essential role in host resistance to GIN infection because they can kill parasitic larvae *in vitro* and *in vivo* (Balic et al., 2006; Rainbird et al., 1998; Terefe et al., 2007). Resistant sheep infected with *H. contortus* have increased levels of eosinophils in abomasal mucosa compared to susceptible sheep (Balic et al., 2006; Gill et al., 2000; Terefe et al., 2009). The number of eosinophils in abomasal tissue in our study was higher in both treated groups than in the Control group at the end of the experiment, but the number was highest in the group supplemented with Se. The number of tissue eosinophils in our previous study did not differ significantly between the control and animals treated with medicinal plants (Mravčáková et al., 2021). We, therefore, concluded that determining the changes in eosinophil numbers at necropsy was inappropriate and that supplementation with medicinal plants did not affect eosinophil levels in the tissues. The difference between this previous experiment and the current experiment may have been due to the higher number of medicinal plants used, re-infection during the experiment, or a longer experimental period. The extended period of plant supplementation may have helped the animals to develop a stronger immune response due to a better nutritional status or higher availability of immunomodulatory bioactive compounds. The increase in the level of tissue eosinophils, however, was larger in the Selplex than the Herbmix group, where 40% of the animals had more than 30% eosinophils scattered in the abomasal mucosa. Few studies have focused on the effect of Se on GIN infections, but to our knowledge, none of the studies investigated the impact of this trace element on immune cells in tissues. We thus had no information to compare with our results. Se, however, is essential for optimal immune function, and higher levels can enhance the function of immune cells and lead to more potent immune responses (McKenzie et al., 1998). We can conclude that supplementation with organic Se in the diet of lambs infected with *H. contortus* can improve local immune responses by increasing eosinophil counts in infected tissue, thus increasing animal resistance to GINs.

Globule leukocytes are derived from mucosal mast cells that migrate into the epithelium (Huntley et al., 1984), and their appearance in the mucosa is typical during nematode infections in both rodents and ruminants (Balic et al., 2000). Their higher numbers have been associated with the resistance of animals to some GIN infections because they secrete leukotrienes and molecules inhibiting larval migration (Douch et al., 1996) and are also responsible for larval rejection (Kemp et al., 2009). Bambou et al. (2013) observed higher infiltration of globule leukocytes in goats resistant to *H. contortus* after primary infection. By comparison, Albuquerque et al. (2019) and Lins et al. (2022) reported stronger abomasal immune responses in infected lambs resistant to haemonchosis, indicated by a large number of globule leukocytes in the abomasal mucosa compared to those susceptible to infection. The infiltration of globule leukocytes in our study was higher in the abomasa of the treated groups than the Control group, as with the tissue eosinophils, but again, the number of these cells was the highest in the group supplemented with Se. Tzamaloukas et al. (2006) reported that lambs infected with *Teladorsagia circumcincta* grazing on chicory (*Cichorium intybus*) and sulla (*Hedysarum coronarium*) had more mucosal mast cells and globule leukocytes compared to lambs grazing on grass (*Lolium perenne*) and clover (*Trifolium repens*). These cell numbers were negatively correlated with worm establishment, suggesting an enhanced local immune response. This effect was attributed to the differences in the nutritional value of the forages, which may also account for the increase in the numbers of these cells in our Herbmix group. The higher numbers of globule leukocytes in the Selplex group could be attributed to an improved local immune response and increased resistance in infected animals due to the immune-enhancing properties of Se, as with eosinophils.

Plasma cells represent the last step in the differentiation of mature B lymphocytes and key components of humoral immunity (Pioli, 2019). Plasma cells produce nematode-specific antibodies during infections, such as IgA, IgG, IgE, and IgM, in response to Th2-associated cytokines, necessary for protective immune responses (McRae et al., 2015). The presence and infiltration of plasma cells in the abomasal mucosa are therefore typical during haemonchosis. Several groups have investigated plasma cells in abomasal tissue associated with haemonchosis. For example, Gill et al. (1994) found an increase in antibody-producing cells (IgA and IgG) in the abomasal mucosa of infected lambs. Muñoz-Guzmán et al.(2012) also observed an increase in plasma cells due to *H. contortus* infection in both the pyloric and fundus parts of the abomasum, but with no significant difference between infected resistant and susceptible breeds. Their results did not associate the number of plasma cells in the abomasum with resistance. Plasma cells in our experiment were mainly scattered in the mucosa in the fundus part of the abomasum and were more numerous in the Herbmix and Selplex groups than the Control group. Similarly, the number of these cells in our previous study was highest in the abomasa of infected lambs treated with a mixture of *A. absinthium* and *M. sylvestris* (Mravčáková et al., 2021). The increase in the number of plasma cells in infected tissue, however, was largest in the Selplex group. Data from another experimental study found that immune responses to infections were weaker in Se-deficient animals than in animals with normal Se levels (Mulhern et al., 1985). The increase in plasma cells was thus probably caused by the immune-enhancing effect of Se supplementation (Dalia et al., 2018).

Lymph nodes are organised lymphoid organs, whose primary function is to filter the tissue and tissue fluids, and are sites of the origin and production of lymphocytes. Lymph nodes should be examined during an infection because they can indicate lesions of the organs and tissues they drain (Elmore, 2006). All animals in our experiment had reactive follicular hyperplasia of the lymph nodes, with follicles of different sizes with germinal centers. This reaction has been found in animals with antigen stimulation, immunisation, or chronic immune stimulation (Maxie, 2015). Pérez et al. (2001) also observed hyperplasia of follicles in goats infected with *H. contortus*. They also observed an enlargement of paracortical areas and medullary cords in infected goats, with hyperplasia of the medullary cords due to a large number of lymphocytes and plasma cells, and numerous eosinophils in the medullary and peritrabecular sinuses, as in our experiment. These histopathological changes in the lymph nodes, however, were caused only by infection. The different dietary treatments in our study did not lead to any significant histopathological changes between groups. Comparing the numbers and types of immune cells in the lymph nodes of infected animals, not only gross histopathology, in the future is therefore necessary to obtain better insights into how different dietary treatments can affect immune responses.

5. Conclusion

This study found that supplementation with a mixture of medicinal plants and organic Se in the diet of lambs infected with the gastrointestinal parasite *H. contortus* for 119 d affected their immune responses during re-infection. This treatment did not induce a strong humoral response in the serum, but it triggered a local cellular response in the abomasum. We can conclude that dietary supplementation with plants or Se probably indirectly affects host resistance by improving their immunity to infection with a parasitic nematode. Further studies, however, are needed to confirm the impact of different diet supplements on the resistance of animals to parasitic infections.

Ethical statement

The experimental protocol was conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences on 14 October 2019 (protocol code 2019/17). Permission to collect samples and carry out the experiments was granted by the participating sheep farmer. Zora Váradyová, on behalf of all of the co-authors

Funding sources

This study was supported by funds from the Slovak Research and Development Agency (APVV 18–0131), the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences (VEGA 2/0008/21), and SAS Programme for PhD students' grants (APP0029). Co-financed within the framework of the Polish Ministry of Science and Higher Education's program: "Regional Initiative Excellence" in the years 2019–2022 (No. 005/RID/2018/19).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to Gabriel Benkovský, Peter Jerga and Valéria Venglovská for laboratory and technical assistance.

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