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Effect of Sainfoin (*Onobrychis viciifolia*) Pellets on Rumen Microbiome and Histopathology in Lambs Exposed to Gastrointestinal Nematodes

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Abstract: Our study analyzed the ruminal fermentation and microbiome, hematological profile, and abomasal histopathology of lambs experimentally infected with a gastrointestinal nematode (GIN) and fed sainfoin pellets (SFPs; 600 g DM/d/animal) for 14 d. Twenty-four lambs infected with *Haemonchus contortus* were divided into two separated groups: animals fed meadow hay (control) and animals fed SFPs. The ruminal contents, fermentation parameters, and microbiome in vitro and in vivo were determined using molecular and microscopic techniques. Ruminal contents in the SFP group indicated smaller populations of *Archaea* ($p < 0.001$), *Methanomicrobiales* ($p = 0.009$), and lower methane concentrations in vitro ($p = 0.046$) and in vivo ($p = 0.030$) than the control group. The relative abundance of *Butyrivibrio fibrisolvens* quantified by real-time PCR was higher in the lambs with the SFP diet ($p = 0.05$). Haemonchosis affected the number of red blood cells of the lambs ($p < 0.001$). The lambs in the SFP group had a higher percentage of damaged abomasa glands than did the control group ($p = 0.004$). The consumption of SFPs by GIN-infected lambs may affect ruminal methanogens and subsequently decrease methane emission without undesirable changes in the ruminal microbiome or the health of the animals.

Keywords: bacteria; flavonoids; hematological profiles; methane concentration; plant bioactive components; ruminal fermentation; sheep

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1. Introduction

Parasitic infection in ruminants, mainly by gastrointestinal nematodes (GINs), influences the intensity of emissions of greenhouse gases and substantially increases the yield of methane emission compared to uninfected animals [1,2]. Promising new nutraceuticals containing bioactive components in ruminant nutrition, however, could have both anthelmintic and anti-methanogenic properties [3,4]. Plant additives with bioactive components can modulate the bacterial, archaeal, and eukaryotic populations in the rumen by interactions

between diet and the microbes because the microbiome plays a crucial functional role in nitrogen use, fermentation, and methane concentration [5].

Plant bioactive components such as flavonoids and condensed tannins (CTs) in feeds have the potential to reduce environmental methane pollution from ruminants by complex bioactivity occurring simultaneously in plants and animals [6]. The main bioactive components in the tanniferous legume sainfoin (*Onobrychis viciifolia*) are flavonoids and CTs formed by the polymerization of flavan-3-ols, with high proportions of prodelphinidins (70%) and procyanidins (30%) [7]. Tanniferous forages are rich in prodelphinidins, have higher antiparasitic activity, and have the effect of reducing methane emissions [8–10]. Sainfoin also reduces the degradation of feed proteins without affecting the digestibility of the nonprotein fraction, thereby increasing the flow of non-ammonia nitrogen and essential amino acids into the small intestine and reducing urinary nitrogen losses [11,12]. The ability of sainfoin to reversibly bind proteins leads to a reduction in GIN parasitism in small ruminants [13,14]. Many studies have focused on the use of sainfoin for its nutritional and anthelmintic effects, but it also contains beneficial flavonoids with similar mechanisms of action as tannins and similarly interferes with the biology of GINs [15]. Combining CTs with quercetin or luteolin identified synergistic anthelmintic effects between tannins and flavonoid monomers [16]. The production of sainfoin pellets (SFPs) at high temperatures and pressure does not affect their bioactivities associated with antioxidative properties [17]. Based on previous studies [18,19], we hypothesized that SFPs would also contribute to desired changes in the ruminal microbiome and histopathology in lambs loaded with parasites.

Analyses of ruminal microbiomes and histopathological observations are needed to identify the possible consequences of bioactive components used in the nutrition of parasite-laden lambs. Our aim was to (1) identify the main flavonoids and phenolic compounds of the SFPs and (2) determine the ruminal fermentation and microbiome, hematological profile, and histopathology of the abomasum of lambs infected with GINs during consumption of SFPs for 14 d.

2. Materials and Methods

2.1. Ethics Statement

This study 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 22 November 2020 (protocol code 2020/21).

2.2. Animals, Diets, and Experimental Design

We housed 24 male lambs (Improved Valachian) 3–4 months of age with an average initial body weight of 15.0 ± 2.07 kg in common stalls for 7 d for a period of adaptation and another 7 d for acclimatizing to feeding, with free access to water. The lambs were obtained from a commercial farm (PD Ružín–Ružín farm, Kysak, Slovakia) where they were also housed during the experiment. Each animal was fed daily meadow hay (MH) *ad libitum* and 300 g dry matter (DM) Mikrop ČOJ, a commercial concentrate (MIKROP, Čebín, Czech Republic). The number of animals used in the experiment was assigned following VICH GL13 guidelines (Veterinary International Committee on Harmonization—Efficacy of anthelmintics: specific requirements for ovine). At the beginning of the experiment—day (D) 0, all parasite-free lambs were infected orally with approximately 5000 third-stage larvae of the MHCo1 strain of *Haemonchus contortus* susceptible to anthelmintics [20]. A modified McMaster technique [21] with a sensitivity of 50 eggs per gram (EPG) of faeces was used for detecting *H. contortus* eggs on D30. The lambs were divided into two groups of twelve animals each (one stall per group) on D30 after infection, when all parasites had matured to the adult stage: control animals fed MH (control, MH, 600 g DM/d/animal) and animals fed sainfoin pellets (SFPs, 600 g DM/d/animal). Both groups continued to be fed commercial concentrate (300 g DM/d/animal). All lambs were positive with a mean

EPG of 9405 ± 4584 in the SFPs group and a mean of 11420 ± 372 in the control group. SFPs were obtained from a commercial source (NATURE'S BEST, EQUOVIS GmbH, Münster, Germany). This feeding scheme continued for 14 d. The lambs were weighed at the end of the experiment and had an average final body weight of 18.3 ± 3.22 kg. All animals were killed at the end of the experiment following the rules of the European Commission (Council Regulation 1099/2009) for slaughtering procedures [22].

2.3. Experiment In Vitro

In vitro gas fermentation technique (IVFT) has been widely used to evaluate the nutritive value of feeds for ruminants and to assess the effect of different nutritional strategies on methane (CH_4) production. Therefore, IVFT using batch-culture incubations of buffered ruminal fluid incubated at 39 °C for 24 h under anaerobic conditions was used [23]. Control animals were donor animals for control groups and SFPs animals were donor animals for SFPs groups for the in vitro experiment. At the end of the experiment the ruminal contents (RCs) were taken from each lamb of each group immediately after slaughter in the abattoir, packed in prewarmed flasks and transported to the laboratory. RCs were pushed through four layers of gauze and pooled in equal volumes based on control and SFP groups. The pooled RCs were purged with CO_2, mixed with McDougall's buffer [24] in a 1:2 ratio, and dispensed in volumes of 35 mL into fermentation bottles (120 mL) containing 250 mg (DM basis) of substrate. The meadow hay or SFPs were used as the substrates of a ration with commercial concentrate (800:200, *w/w*) as the components of the diets for the controls and SFP groups for in vitro experiment. Commercial concentrate, MH, and SFPs were ground using a grinder (Molina, MIPAM, České Budějovice, Czech Republic) and sieved through 0.15–0.40 mm screens. The in vitro experiment had a completely randomized design using the two diets (control and SFP) in fermentations with the two inocula of ruminal fluids (control and SFPs), with three replicates (three incubation bottles) for each diet and inoculum. The in vitro experiment was repeated three times within three consecutive days ($n = 3 \times 3$).

2.4. Chemical Analysis of the Dietary Substrates

The chemical compositions of the dietary substrates (Table 1) were analyzed using standard methods [25,26].

Table 1. Chemical compositions of the dietary substrates.

Substrate	DM (g/kg)	NDF (g/kg DM)	ADF (g/kg DM)	CP (g/kg DM)	N (g/kg DM)	Ash (g/kg DM)
Meadow hay	885	640	423	84	14	84
Concentrate	888	231	130	211	34	104
SFPs	918	460	357	121	19	100

DM, dry matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; N, nitrogen; SFPs, sainfoin pellets.

2.5. Analysis of Bioactive Compounds

SFPs were ground to a fine powder, and 100 mg were extracted three times with 80% MeOH at 40 °C for 60 min. The extracts were evaporated to dryness and were then dissolved in 2 mL of Milli-Q water (acidified with 0.2% formic acid) and purified by solid-phase extraction using an Oasis HLB 3 cc Vac Cartridge (Waters Corp., Milford, CT, USA) as was previously described [27]. Bioactive compounds were analyzed by ultrahigh resolution mass spectrometry (UHRMS) on a Dionex UltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector connected to a high-resolution quadrupole time-of-flight mass spectrometer (Compact, Bruker Daltonik GmbH, Bremen, Germany). Phenolic acid and flavonoids were identified chromatographically on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, Torrance, CA, USA), with mobile phase A consisting of 0.1% (*v*/*v*) formic acid in water and mobile phase B consisting of 0.1% (*v*/*v*) formic acid in acetonitrile, as was previously described [19]. Stock solutions of hyperoside and chlorogenic acid were prepared in 80% MeOH at concentrations of 2.5 and 3.6 mg/mL,

respectively, and kept frozen until used. Calibration curves for these two compounds were constructed based on seven concentration points (from 500 to 3.6 µg/mL). Hyperoside was used to calculate the number of flavonoids identified in the extract, and chlorogenic acid was used for phenolic acids, using Bruker QuantAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany). All analyses were performed in triplicate.

2.6. Basic Ruminal Fermentation Analysis

RC samples from the in vitro and in vivo experiments were collected for determining pH, methane, volatile fatty acids (VFAs), ammonia concentrations, in vitro DM digestibility (IVDMD), and population of ruminal microorganisms (bacteria, protozoa, and methanogens). Concentrations of methane in vitro and VFAs were determined by gas chromatography on a PerkinElmer Clarus 500 gas chromatograph (Perkin Elmer, Inc., Shelton, CT, USA) [28]. Methane concentration in vivo was calculated by measuring the molar proportions of the VFAs in the rumen as: 57.5 mol glucose = 65 mol acetate + 20 mol propionate + 15 mol butyrate + 60 mol CO_2 + 35 mol CH_4 + 25 mol H_2O [29]. The concentration of ammonia-N was determined using the phenol-hypochlorite method [30].

2.7. Rumen Microbial Quantification

Samples for counting ciliate protozoa from the RCs were fixed in equal volumes of 8% formaldehyde, and the protozoa were counted and identified microscopically [31]. Total bacteria, *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* from the in vitro experiment and *Archaea* and *Methanobacteriales* from the in vivo experiment were quantified using fluorescence in situ hybridization as described previously [32]. DNA for quantifying bacteria was isolated from the ruminal samples using a Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) to lyse the cells [33] followed by purification using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA concentrations and qualities were measured using a NanoPhotometer R NP80 (Implen GmbH, München, Germany). Total bacteria, *Streptococcus bovis*, *Butyrivibrio proteoclasticus*, *B. fibrisolvens*, *Fibrobacter succinogenes*, *Megasphaera elsdenii*, *Ruminococcus albus*, *R. flavefaciens*, and the genera *Prevotella and Lactobacillus* were quantified by real-time PCR using the PCR primers [34–39].

2.8. Hematological Parameters

Samples of blood were collected from the jugular vein of each animal on D0, D23, D30, D37, and D44. Basic hematological parameters were determined using an Abbott CELL-DYN 3700 hematological analyzer (Global Medical Instrumentation, Inc., Ramsey, NJ, USA).

2.9. Histopathology

Samples of fresh abomasal tissues were washed in a phosphate buffer (0.1 M, pH 7.4), put in plastic containers, and fixed in a 10% buffered FA solution as pieces of tissue spread on a flat piece of polystyrene as previously described [19]. The fixed material was processed using a series of reagents in the following sequence: 75% alcohol for 1 h, 90% alcohol for 1 h, 95% alcohol for 1 h, 100% alcohol 3 times for 1 h. Then, the material was cleared in xylene 3 times for 1 h. The material was infiltrated in paraffin 3 times for 1 h 20 min. The described steps took place in a tissue processor (Excelsior AS Thermo Scientific, Runcorn, UK). Afterward, specimens were embedded in Paraplast PLUS paraffin blocks (Leica, Buffalo Grove, IL, USA), which were then cut with a rotary microtome into sections 2.5 µm thick. Slides with a paraffin section were automatically stained with hematoxylin and eosin (Varistain Gemini Thermo Scientific, Runcorn, UK). An Axio Lab. A1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam ERc 5s digital camera was used for histological evaluation. Photographs were analyzed and recorded using ZEN 2.3 (blue edition) software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany, 2011).

2.10. Statistical Analysis

Data for the fermentation parameters and microbial populations were analyzed using an unpaired *t*-test (GraphPad Prism 8; GraphPad Software, Inc., San Diego, CA, USA). Two–way analyses of variance were used for analyzing the hematological parameters as models representing the two animal groups (control and SFPs) and sampling days (D0–D44). The effects included in the model were treatment (T), time, and the interaction between them (T × time). Results were considered significant at $p < 0.05$.

3. Results

3.1. Phytochemicals

The phytochemical substances in the SFPs consisted of 32.56 g/kg DM flavonoids, 4.68 g/kg DM phenolics, and 0.37 g/kg DM others (glycosides, hydroxy fatty acids, and a derivative of cinnamic acid, Table 2).

Table 2. The main phytochemicals in the MeOH extract of the sainfoin pellets.

No.	RT (min)	UV (nm)	m/z [M-H]$^-$	MS ^{2} Main Ion	MS ^{2} Fragments	Formula	Compound	mg/g DM
1	2.4	261,296	153.018	109.0291		$C_7H_6O_4$	3,5-Dihydroxybenzoic acid	0.26
2	2.6	261,296	153.018	109.0291		$C_7H_6O_4$	2,4-Dihydroxybenzoic acid	0.29
3	4.6	245sh,348	175.0601	157.0488	131,115	$C_7H_{12}O_5$	3-Isopropylmalic acid	0.28
4	5.1	248sh,326	353.0884	191.0557		$C_{16}H_{18}O_9$	Trans-5-caffeoylquinic acid	0.37
5	5.9		367.1034	193.0501	173.134	$C_{17}H_{20}O_9$	3-*O*-Caffeoyl-4-*O*-methylquinate	0.24
6	6.2	282,316	239.0563	179.034	195,221,149	$C_{11}H_{12}O_6$	Derivative of cinnamic acid	0.09
7	6.7	269,348	771.1993	609.1451	462,301	$C_{33}H_{40}O_{21}$	Quercetin 3-rutinoside 7-galactoside	0.26
8	8.6	269,345	355.1042	161.0227	193.179	$C_{16}H_{20}O_9$	Trans-feruloylglucose	0.25
9	9.4	269,340	625.1400	316.0221	117.163	$C_{27}H_{30}O_{17}$	Myricetin-3-rutinoside	1.91
10	9.5	280	325.0937	119.0497	117.163	$C_{15}H_{18}O_8$	8-β-Glucopyranosyloxycinnamic acid	3.27
11	9.8	266,346	623.1262	285.0408	447	$C_{27}H_{28}O_{17}$	Luteolin 4′-glucoside 7-galacturonide	1.80
12	9.9	262,345	739.2094	284.0327	572,255,178	$C_{33}H_{40}O_{19}$	Kaempferol 3-(2″-rhamnosylrutinoside)	0.32
13	10.2	265,349	339.1092	145.0276	163	$C_{16}H_{20}O_8$	TPCA [1]	0.27
14	10.5	256,354	609.1462	300.0281		$C_{27}H_{30}O_{16}$	Rutin	18.92
15	10.8	269,354	463.0912	300.0269	271,151	$C_{21}H_{20}O_{12}$	Quercetin-4′-glucoside	1.06
16	10.9	269,343	637.1414	299.0567	284.2337	$C_{28}H_{30}O_{17}$	MHGB [2]	2.03
17	11.0	264,346	447.0930	285.0397	269,209,251	$C_{21}H_{20}O_{11}$	Kaempferol 3-*O*-glucoside	0.64
18	11.4	265,344	593.1527	285.0408	151,327,178	$C_{27}H_{30}O_{15}$	Kaempferol 3-*O*-glucoside [3]	1.87
19	11.6	254,354	623.1626	315.0515	151,243,271	$C_{28}H_{32}O_{16}$	Isorhamnetin 3-*O*-rhamnoglucoside	3.75

[1] Trihydroxy-6-[4-(3-oxobutyl)phenoxy]oxane-2-carboxylic acid; [2] 2-(3-Methoxy-4-hydroxyphenyl)-5-hydroxy-7-[2-*O*-(beta-D-glucopyranuronosyl)-beta-D-glucopyranosyloxy]-4H-1-benzopyran-4-one; [3] Kaempferol 3-*O*-glucoside-7-rhamnoside.

3.2. Ruminal Fermentation In Vitro

Methane concentration was significantly lower ($p = 0.046$), but IVDMD ($p < 0.001$), total VFA concentration ($p = 0.011$), and *n*-valerate concentration ($p = 0.017$) were significantly higher, in the SFP than the control group (Table 3). *Archaea* and *Methanomicrobiales* populations were smaller in the SFP than the control group ($p < 0.001$ and $p = 0.009$, respectively).

Table 3. Effect of SFPs on ruminal fermentation and microbial populations in vitro.

	Control	SFP	SEM	*p*
Ammonia N (mg/L)	270	282	15.5	0.849
Methane (mmoL)	1.15	1.02	0.063	0.046
pH	7.60	7.45	0.041	0.064
IVDMD (g/kg DM)	311	447	18.0	<0.001
Total VFAs (mmoL/L)	31.5	39.9	1.76	0.011
Acetate (mol%)	65.5	66.1	0.997	0.768
Propionate (mol%)	17.7	17.1	0.799	0.711

Table 3. *Cont.*

	Control	SFP	SEM	*p*
n-Butyrate (mol%)	11.0	11.2	0.239	0.764
iso-Butyrate (mol%)	0.861	0.617	0.158	0.455
n-Valerate (mol%)	2.25	2.56	0.066	0.017
iso-Valerate (mol%)	2.50	2.27	0.115	0.318
Caproate (mol%)	0.150	0.181	0.019	0.430
Acetate: propionate	4.02	4.10	0.353	0.923
Total protozoa (10^3/mL)	6.65	8.7	6.612	0.438
Total bacteria (10^8/mL)	4.02	3.9	0.164	0.721
Archaea (10^6/mL)	3.93	2.64	0.240	<0.001
Methanobacteriales (10^6/mL)	1.53	1.48	0.024	0.363
Methanomicrobiales (10^5/mL)	1.56	1.42	0.027	0.009

IVDMD, in vitro dry matter digestibility; VFAs, volatile fatty acids.

3.3. Ruminal Fermentation and Microbiota in the Lambs

The SFPs significantly affected ($p = 0.030$) methane concentrations in the lambs (Table 4). Total protozoa, expressed as a count per gram of wet RC (wRC) in the lambs, did not differ significantly ($p = 0.05$) between the groups. The populations of *Archaea* ($p < 0.001$), *Methanomicrobiales* ($p = 0.009$), and *Methanobacteriales* ($p < 0.001$) were significantly smaller for the SFP than the control group. The relative abundances of *S. bovis*, *B. proteoclasticus*, *F. succinogenes*, *M. elsdenii*, *R. albus*, *R. flavefaciens*, and the genera *Prevotella* and *Lactobacillus* did not differ significantly between the groups ($p = 0.05$). The relative abundance of *B. fibrisolvens* was significantly higher in the SFP than in the control group ($p = 0.006$). The relative abundance of the 16S rRNA gene was expressed as an arbitrary unit (AU) relative to the total abundance of bacterial genes of the control group.

Table 4. Effect of SFPs on ruminal fermentation and microbial populations in the lambs.

	Control	SFP	SD	*p*
Ammonia N (mg/L)	205	199	79.1	0.849
Methane (mmoL)	3.17	2.30	0.903	0.030
pH	6.36	6.37	0.575	0.974
Total VFAs (mmoL/L)	68.2	70.2	27.71	0.847
Acetate (mol%)	74.6	73.3	4.33	0.462
Propionate (mol%)	11.9	10.5	4.42	0.425
n-Butyrate (mol%)	11.1	13.5	3.96	0.123
iso-Butyrate (mol%)	0.06	0.06	0.140	0.946
n-Valerate (mol%)	1.33	1.60	0.828	0.414
iso-Valerate (mol%)	0.89	0.77	0.595	0.633
Caproate (mol%)	0.20	0.34	0.473	0.457
Acetate: Propionate	7.19	7.91	0.529	0.507
Total protozoa (10^5/g wRC)	6.7	8.2	5.61	0.508
Archaea (10^7/mL)	7.71	4.79	1.84	<0.001
Methanobacteriales (10^7/mL)	3.74	2.90	0.672	<0.001
Methanomicrobiales (10^7/mL)	3.50	2.50	0.463	0.009
Total bacteria (AU)	1.0	1.01	0.110	0.863
S. bovis (AU)	1.0	1.04	0.145	0.525
B. proteoclasticus (AU)	1.0	1.06	0.161	0.325
B. fibrisolvens (AU)	1.0	1.13	0.124	0.006
F. succinogenes (AU)	1.0	0.97	0.311	0.832
M. elsdenii (AU)	1.0	0.94	0.258	0.587
R. albus (AU)	1.0	1.10	0.175	0.160
R. flavefaciens (AU)	1.0	0.97	0.147	0.594
Genus *Prevotella* (AU)	1.0	0.96	0.299	0.729
Genus *Lactobacillus* (AU)	1.0	1.04	0.126	0.500

wRC, count per gram of wet ruminal content; AU, arbitrary unit.

3.4. Hematological Parameters

The complete red blood cell (RBC) hemograms of each infected animal identified clinical signs of haemonchosis such as anemia after D23 (Table 5). The RBC count, level of hemoglobin (HGB), hematocrit (HCT), and mean corpuscular volume (MCV) were not significantly influenced by treatment or the interaction between treatment and time ($p = 0.05$). Time significantly affected all RBC parameters ($p < 0.001$). Total leukocytes, neutrophils, and lymphocytes were not significantly influenced by treatment, time, or the interaction between them ($p = 0.05$, Table 6). Treatment and time significantly affected the counts of monocytes ($p = 0.05$) and basophils ($p = 0.05$ and $p = 0.01$, respectively), and time significantly affected the counts of eosinophils ($p < 0.001$).

Table 5. Effects of SFPs on the red blood cell parameters of the lambs.

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
RBC (T/L)	0	11.37	11.63	1.176	0.133	<0.001	0.681
	23	7.68	7.10	1.289			
	30	7.22	6.56	1.382			
	37	7.15	6.55	1.373			
	44	7.00	6.83	1.276			
Hemoglobin (g/L)	0	111.7	115.5	11.62	0.326	<0.001	0.462
	23	74.6	68.9	11.30			
	30	71.5	64.8	12.89			
	37	72.0	67.1	14.28			
	44	69.5	71.7	14.29			
Hematocrit (g/L)	0	0.263	0.265	0.0251	0.208	<0.001	0.458
	23	0.187	0.171	0.0261			
	30	0.182	0.165	0.0313			
	37	0.185	0.174	0.0339			
	44	0.178	0.187	0.0336			
MCV (fL)	0	23.2	23.0	1.86	0.281	<0.001	0.354
	23	24.5	24.4	2.11			
	30	25.4	25.3	2.25			
	37	26.0	26.7	2.04			
	44	25.6	27.4	2.29			

RBC, red blood cell; T/L, 10^{12} per liter; MCV, mean corpuscular volume; SD, standard deviation.

Table 6. Effects of SFPs on the total leukocyte and differential counts of the lambs.

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
Total leukocyte (G/L)	0	8.68	8.92	2.280	0.159	0.225	0.951
	23	8.00	9.25	2.515			
	30	7.24	7.81	4.475			
	37	7.29	7.71	1.705			
	44	6.55	7.90	2.047			
Neutrophils (G/L)	0	3.18	3.10	1.662	0.361	0.304	0.923
	23	3.13	3.66	1.589			
	30	2.69	2.84	1.064			
	37	2.63	2.68	1.095			
	44	2.51	3.01	1.526			

Table 6. *Cont.*

	Day	Control	SFP	SD	*p*		
					Treatment (T)	Time	T × Time
Lymphocytes (G/L)	0	2.23	2.22	1.313	0.899	0.606	0.987
	23	1.95	2.13	0.977			
	30	2.35	2.19	1.045			
	37	2.61	2.50	1.186			
	44	2.21	2.18	1.214			
Monocytes (G/L)	0	2.38	2.55	1.288	0.047	0.024	0.977
	23	2.10	2.48	1.055			
	30	1.87	2.30	0.983			
	37	1.67	1.92	0.606			
	44	1.44	1.93	0.686			
Eosinophils (G/L)	0	0.093	0.096	0.0596	0.200	<0.001	0.395
	23	0.046	0.041	0.0193			
	30	0.065	0.033	0.0356			
	37	0.043	0.033	0.0177			
	44	0.039	0.041	0.0225			
Basophils (G/L)	0	0.803	0.945	0.7947	0.028	0.002	0.930
	23	0.778	0.953	0.6667			
	30	0.275	0.445	0.2770			
	37	0.336	0.575	0.4151			
	44	0.349	0.751	0.5041			

SD, standard deviation; G/L, 10^9 per liter.

3.5. Histopathology

The changes in the abomasal tissues of the lambs inoculated with *H. contortus* were mild and included damage to epithelial cells and inflammatory infiltration (Table 7). The percentage of damaged glands ($p = 0.004$) was significantly higher, and mucosal hypertrophy (not significant, $p = 0.05$) was marginally higher, in the SFP than in the control group. Glandular dilatation and submucosal edema were similar in both groups.

Table 7. Effects of SFPs on the histopathology of the abomasal tissues of the lambs.

Effect	Control	SFP	SD	*p*
Hypertrophy of mucosa (%)	15.4	30.8	3.31	0.375
Epithelial cell damage (%)	100	100	0.00	–
Hyperplasia of mucus-producing cells (%)	7.7	0	1.51	0.327
Dilatation of glands (%)	30.8	30.8	3.62	0.999
Damage of glands (%)	0	46.2	3.31	0.004
Inflammatory cell infiltration (%)	100	100	0.00	–
Submucosal edema (%)	30.8	38.5	3.73	0.695

4. Discussion

Multidisciplinary studies (agronomic, nutritional, parasitological, and chemical) have identified many benefits to animal health of tanniferous forages and legumes used as feed for ruminants [40,41]. Sainfoin generally contains mainly CTs (approximately 42–50 g CTs/kg DM), which have been well studied [42–44]. The consumption of sainfoin disturbs various stages of parasitic life cycles, mainly due to its high tannin content [45]. The anthelmintic activity of CTs, however, can be increased by the addition of flavonoids, which also interfere with the biology of GINs [15,16]. We therefore focused on the analysis of flavonoids and phenolic acids.

Quantitative UHRMS analyses of the bioactive components in the SFPs identified more than 32.0 g/kg DM flavonoids and 4.5 g/kg DM phenolic acids. The main flavonoid was rutin (18.92 mg/g DM), which has multiple pharmacological activities with metabolic

health benefits [46]. Rutin can alter the ruminal microbiome and reduce the population of methanogenic bacteria [47]; and adding rutin (3.0 mg/kg) to feed dairy cows for 11 weeks improved the efficiency of carbohydrate fermentation in the rumen and the ability to synthesize protein [48]. Our results with SFPs indicated reductions in methane concentrations and *Archaea* population sizes of 11 and 33% and 27 and 38% in in vitro and in vivo treatments, respectively. Ruminal contents in the SFP group indicated also smaller populations of *Methanomicrobiales* (in vitro and in vivo) and *Methanobacteriales* (in vivo) than the control group. In another study, dietary supplementation with dry fumitory, mallow, wormwood, and chamomile with flavonoids (0.4–12.2 g/kg DM) for 70 d had no anti-methanogenic effect in lambs [19]. The anti-methanogenic effect of SFPs observed in our study may have been due to the direct effect of either the CTs [6,49] or rutin [50] or both on methanogenesis in the SFP group. Data on reducing methane concentration by rutin supplementation in vitro and in vivo, however, have been inconsistent [51–53]. Acceptably low methane emissions can therefore be achieved by a suitable choice of the vegetative stage of sainfoin [54]. The stimulation of gas concentration by rutin (50 mg/g DM) in ruminal fermentation in vitro led to an increased CO_2 concentration and a decreased methane concentration, probably because rutin is a substrate for nonmethanogenic microbiota [50]. However, the consumption of SFPs by infected lambs for 14 d in our experiment affected ruminal methanogens and consequently reduced methane emission without adverse changes in the ruminal microbiome.

The relative abundance of *B. fibrisolvens* in our study was higher in the SFP than the control group, because the replacement of MH by SFPs was probably associated with the increased demand for microbial degradation of fiber in the SFP group. Other bacterial species were not significantly affected, probably due to the relatively short SFP treatment. *B. fibrisolvens* plays an important role in the ruminal fermentation of polysaccharides that participate in cellulolytic processes in the rumen but do not have an autonomous cellulolytic capacity [55]. Changes in the relative abundance of *B. fibrisolvens* suggest that the SFPs did not negatively affect ruminal fermentation (e.g., total and individual VFAs). A diet with plant bioactive compounds can affect the ruminal microbiome, the kinetics of fermentation, and the response and adaptation to anti-methanogenic compounds, sometimes leading to inconsistent efficacy of phytochemicals [56–58]. We cannot sufficiently confirm the effect of SFPs on ruminal fermentation in lambs, because the SFPs were consumed for only 14 d. We also observed no effects of short-term SFP feeding on total protozoal counts in vitro and in vivo. The in vitro experiments on the effects of CT fractions of differing molecular weights on *Leucaena leucocephala* identified a lowering of the total number of ciliate protozoa with changes in counts of community members [59]. Similar effects were observed in vivo after long-term feeding of lambs with extracts of *Acacia negra* and *Uncaria gambir*, sources rich in CTs [60]. The feeding of dry leaves of *L. leucocephala* (12–36% of DM intake), however, did not affect ruminal protozoan, bacterial, or archaeal populations in crossbred heifers [61]. Supplementation of rutin for three weeks in dairy cows (3.0 mg/kg diet) did not significantly decrease counts of ruminal protozoa [49]. The amount and composition of the CTs and the length of treatment are likely the main factors influencing the effects of CT on the ruminal microbiome. In our experiment, even feeding only SFPs did not affect the protozoan counts. We can speculate about the amount of intake of CTs in the diet of the control group. Several studies have reported the CT compositions of various kinds of forages and MH from permanent pastures [6,62]. Unfortunately, we did not measure the CT content of the control MH diet.

Our study confirmed a significant reduction in RBC count, HGB level, and HCT from D23 in both groups of lambs infected with *H. contortus*, consistent with our previous results [20,28,63]. The reductions were likely due to damage caused by the GIN parasites, but SFPs as a replacement for hay did not affect the RBC parameters. The intensity and duration of hematological disorders depend on the nutritional status of infected sheep because protein-enriched diets induce resistance to infection associated with the improved regenerative capacity of bone marrow [64]. In addition to the nutritional status of the

host, differences in RBC disorders during GIN infection may be affected by the species of nematode, the severity of the infection, the iron stores and bodily reserves of the host, and the susceptibility of the host breed [65]. The basophil level in our study was higher in the infected lambs of the SFP group. Basophils are generally relatively rare and short-lived cells and probably played an important role in the immune response in the SFP group to GIN infection in our experiment [66]. The number of monocytes, eosinophils, and basophils was differentially affected by the time after infection, consistent with the blood variables during a subclinical *H. contortus* infection [67].

The development of GINs in host abomasa causes pathology, with mucosal damage and gastropathy with protein loss, followed by inflammatory immune responses of the host [68]. Our observations included microscopic changes in the abomasa, such as mucosal hypertrophy, damage to epithelial cells, mucus-producing cell hyperplasia, glandular dilatation, glandular damage, inflammatory cell infiltration, and submucosal edema. A roughened and hyperemic abomasal mucosa with enlarged glands and globular leukocytes have been described in lambs infected with *H. contortus* [69]. In our experiment, glandular damage in the SFP group differed significantly between the experimental groups, but the other changes in the abomasal mucosa were essentially the same for both infected groups. Mucosal hypertrophy was also more pronounced but not significant in the lambs fed with SFPs. An increased percentage of abomasal injuries and mucosal hypertrophy in the SFP group was attributed to regeneration, which is more common in abomasal tissue due to herbal treatment [70]. The histopathological changes in lambs in our previous study infected with *H. contortus* and supplemented with dry *Artemisia absinthium* and *Malva sylvestris* were predominantly on the mucosal membrane, with inflammatory cell infiltrates (mainly lymphocytes and macrophages with a mixture of eosinophils, plasma cells, and mast cells) [70]. Subclinical *H. contortus* infections generally damage the abomasal mucosa, which was very similar in the two infected groups, i.e., with and without SFPs.

5. Conclusions

Replacing MH with SFPs affected the composition of methanogenic bacteria in the rumens of the lambs and consequently reduced methane emissions, thus helping to reduce the environmental burden of methane and minimizing the adverse effects on animal health. From this point of view, the use of SFPs can be very useful in good agricultural practice. This study, to the best of our knowledge, is the first on the effect of SFPs on the ruminal microbiome and the abomasal histopathology of lambs loaded with parasites.

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