Article

Not peer-reviewed version

Growth Performance, Nutrient Composition, Ruminal Microbiota, and Fermentation In Vivo and In Vitro Associated with Endoparasites of Lambs

Klára Mikulová , Daniel Petrič , Michaela Komáromyová , Dominika Batťányi , Martyna Kozłowska , Adam Cieslak , Sylwester Ślusarczyk , Marián Várady , Zora Váradyová *

Posted Date: 20 July 2023

doi: 10.20944/preprints202307.1365.v1

Keywords: Archaea; ciliated protozoa; Haemonchus contortus; ruminal fermentation parameters; methane emission

Article

Growth Performance, Nutrient Composition, Ruminal Microbiota, and Fermentation In Vivo and In Vitro Associated with Endoparasites of Lambs

Klára Mikulová [1,2], Daniel Petrič [1], Michaela Komáromyová [3], Dominika Batťányi [1], Martyna Kozłowska [4], Adam Cieslak [4], Sylwester Ślusarczyk [5], Marián Várady [3] and Zora Váradyová [1,*]

[1] Centre of Biosciences of Slovak Academy of Sciences, Institute of Animal Physiology, Šoltésovej 4-6, 040 01 Košice, Slovakia; mikulova@saske.sk (K.M.); petric@saske.sk (D.P.); mravcakova@saske.sk (D.B.)
[2] University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81, Košice, Slovakia
[3] Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice, Slovakia; komaromyova@saske.sk (M.K.); varady@saske.sk (M.V.)
[4] Department of Animal Nutrition, Poznan University of Life Sciences, Wolynska 33, 60-637 Poznan, Poland; martyna.kozlowska@up.poznan.pl (M.K.); adam.cieslak@up.poznan.pl (A.C.)
[5] Department of Pharmaceutical Biology and Botany, Wroclaw Medical University, Borowska 211, 50-556 Wroclaw, Poland; sylwester.slusarczyk@umed.wroc.pl
* Correspondence: varadyz@saske.sk; Tel.: +421-55-792-2972

Abstract: We conducted an experiment on lambs to determine the effect of infection with the gastrointestinal nematode (GIN) *Haemonchus contortus* on growth performance, nutrient composition, microbiota, and ruminal fermentation in vivo and in vitro. Twelve female lambs were equally divided into two groups: uninfected animals (CON) and animals infected with approximately 5000 third-stage ISE larvae derived from the MHCo1 strain of *H. contortus* (INF). Two lambs per group were killed on days 48, 49, and 50 after infection and ruminal content was collected separately from each lamb. Batch cultures of ruminal fluid from CON and INF were incubated in vitro with 0.25 g meadow hay, a plant mixture and chicory used as substrates. The ruminal populations of protozoa, bacteria, total Archaea, and the archaean orders *Methanobacteriales* and *Methanomicrobiales* were significantly higher in INF than CON. In vivo concentrations of n-butyrate, iso-butyrate, and iso-valerate were significantly lower in INF than CON. In vitro values after 24 h of ruminal fermentation varied within the substrate. GIN infection by *H. contortus* was associated with increased methanogenesis. In conclusion, chicory was a promising substrate for mitigating methane production and may modulate the ruminal microbiome of GIN-infected lambs due to its chemical and phytochemical composition.

Keywords: *Archaea*; ciliated protozoa; *Haemonchus contortus*; ruminal fermentation parameters; methane emission

1. Introduction

The parasitic worms of ruminants, such as gastrointestinal nematodes (GINs), influence several factors associated with methane emissions, including feed efficiency, nutrient use, and animal production. Interest in research on gastrointestinal (GI) parasitism associated with ruminal fermentation and methanogenesis in ruminants is growing in Europe because ruminant GINs are very economically important [1]. Recent studies have reported that GI parasitism in ruminants increases the emission of greenhouse gases and mostly affects the primary factors (the ruminal microbiome and the intake and composition of feed) responsible for enteric methane emissions [2–4]. An association between infection by *Haemonchus contortus* and the diversity of the microbial communities of the abomasa and rumens of sheep from seven to 50 d *post*-infection has been confirmed [5].

 doi:10.20944/preprints202307.1365.v1

The ability of ruminants to convert polysaccharides in plant cell walls into meat and milk is due to a symbiotic association of a complex microbial community (bacteria, methanogenic archaea, anaerobic fungi, and protozoa) in the rumen. These microbial communities produce various enzymes that convert ingested feed into short-chain fatty acids (FAs) and microbial proteins that the animal uses for growth. Due to its great diversity, the ruminal microbiome is responsible for supplying ruminants with their dietary and metabolic needs [6]. Ruminal microbial communities, however, can be influenced by several host factors under specific conditions, such as age, breed, disease, infection, feed, and additives [7]. GIN infections can lead to substantial changes in the digestive tract that disrupt interactions between hosts and their gut microbiomes because products secreted by GINs affect the growth and metabolism of the resident microbial communities [8,9]. Changes in the composition of nutrients can also alter the composition of the ruminal microflora and its enzymatic activity [10]. Plant bioactive compounds can have antimicrobial, antiparasitic, anti-inflammatory, antioxidant, and immunological properties [11,12], and their diversity, synergy, and various combinations may contribute to pharmacological efficacy in GIN infections [13–15].

Little information is available on the effects of GI parasitism on ruminal fermentation and methanogenesis, so we hypothesised that plants with antiparasitic properties would also influence the ruminal microbial communities of GIN-infected lambs. The use of mallow, chamomile, Jacob's ladder, wormwood, and chicory in the feed of ruminants provides high-quality roughage with a high content of crude proteins (from 140 to 160 g/kg dry matter), which increases the weight gain of lambs [16–18]. We used the traditional medicinal plants mallow, chamomile, fumitory, wormwood, and chicory in vitro based on their antiparasitic properties [13,19] to investigate the effect of (1) *H. contortus* infection on ruminal fermentation, microbiota, and microbial specific enzymatic activity in lambs, and (2) a plant mixture Herbmix (mallow, chamomile, fumitory, and wormwood) and the medicinal plant chicory on in vitro ruminal fermentation using inocula from infected animals.

2. Materials and Methods

2.1. Ethical Study

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 16 February 2022 (protocol code 2022/09).

2.2. Animals, Diets, and Experimental Design

Twelve female Improved Valachian lambs, 3-4 months of age with an average initial body weight of 17.7 ± 2.12 kg, were housed in common stalls on a sheep farm (PD Ružín–Ružín farm, Kysak, Slovakia) with free access to water. The animals were dewormed with the recommended dose of albendazole (Albendavet 1.9% susp. a.u.v, Divasa-Farmavic S.A., Barcelona, Spain) 10 d before the start of the trial and were kept indoors to maintain parasite-free conditions. Each animal was fed daily meadow hay (MH) *ad libitum* and 300 g dry matter (DM) of Mikrop ČOJ, a commercial concentrate (Mikrop, Čebín, Czech Republic). The lambs were divided into two groups of six animals each (one stall per group): control (CON) and infected (INF). Adequate access to water and feeder space was provided for each animal. At the beginning of the experiment, six parasite-free lambs were infected orally with approximately 5000 third-stage ISE larvae derived from the MHCo1 strain of *H. contortus* susceptible to anthelmintics [20]. A modified McMaster technique [21], with a sensitivity of 50 eggs per gramme (EPG) of faeces was used for detecting *H. contortus* eggs six weeks after experimental infection. Two lambs per group were killed on days 48, 49, and 50 after infection following the rules of the European Commission (Council Regulation 1099/2009) for slaughtering procedures [22].

 doi:10.20944/preprints202307.1365.v1

2.3. Experiment In Vitro

Ruminal and abomasal contents were collected from the slaughtered CON and INF lambs. Both contents were separately passed through four layers of gauze, mixed at a 1:2 ratio with McDougall's buffer [23], and dispensed in volumes of 35 ml into serum bottles containing 0.25 g of a substrate for subsequent use as inocula. Meadow hay (MH), a dry herbal mixture (Herbmix), and dry chicory (*Cichorium intybus* L.) were used as substrates. Herbmix contains flowers of mallow (*Malva sylvestris* L.) and chamomile (*Matricaria chamomilla* L.) and stems of fumitory (*Fumaria officinalis* L.) and wormwood (*Artemisia absinthium* L.) mixed in equal proportions. The plants obtained from a commercial source (Agrokarpaty, Plavnica, Slovak Republic) were ground through a 0.15-0.40 mm screen using a Molina grinder (Mipam bio s.r.o., České Budějovice, Czech Republic) or a stand mixer (Bosch, Berlin, Germany). The treatments were based on the inocula: inoculum from CON or INF with MH, and inoculum from CON or INF with Herbmix or chicory (Herbmix/Chicory). Clumps of adult worms were manually collected from the abomasal contents of infected lambs, and approximately 20-30 adult worms were placed into the INF serum bottles. The experiment was carried out using the in vitro gas-production technique (IVGPT) on batch culture incubations of buffered ruminal fluid (RF) incubated for 24 h at 39 °C under anaerobic conditions. Three replicates (three serum bottles) were prepared for the substrates (MH, and Herbmix/Chicory) and each inoculum. The experiment was arranged in a 3 × 2 × 3 completely randomised design using the three substrates in fermentations with two inocula (CON and INF) and repeated three times within three days (n = 3 × 3).

2.4. Ruminal Measurements

2.4.1. Ruminal Fermentation and Microbial Quantification

Samples of ruminal contents from the CON and INF lambs were collected for determining pH and ammonia-N concentration and identifying and quantifying the short-chain FAs (SCFAs) and the populations of ruminal microorganisms. The volume of accumulated gas released from the recorded pressure, or the volume of gas produced after 24 h of fermentation, was determined using IVGPT [24]. Gas samples (1000 µL) were collected from the headspace of the bottles using a gastight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) for measuring the methane concentration in the samples. The SCFAs and methane were analysed on a PerkinElmer Clarus 500 gas chromatograph (Perkin Elmer, Shelton, USA) [25]. The concentration of ammonia-N was determined using the phenol-hypochlorite method [26]. Samples for counting ciliate protozoa from the ruminal fluid were fixed in equal volumes of 8% formaldehyde, and the protozoa were counted and identified microscopically [27]. Bacteria, total *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* in the ruminal contents were quantified using fluorescence in situ hybridisation [28].

2.4.2. Specific Enzymatic Activities

The specific enzymatic activities for the ruminal microorganisms were determined by the preparation of a cell-free homogenate. The ruminal content, which was immediately frozen at -80 °C after collection, was thawed before homogenisation and diluted in an amount of approximately 6 g with 2 mL of a phosphate-citrate buffer solution (pH 6.8) with cOmplete ™ mini EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The contents were homogenised in an ice bath using a 4710 series ultrasonic homogeniser (Merck KGaA, Darmstadt, Germany) at a power of 80 W pulsed in one-minute intervals (homogenisation and cooling). All samples were centrifuged at 16 000 × g for 15 min, and the supernatant was used for the enzymatic assays. The specific enzymatic activities of amylase, cellulase, and xylanase were determined colorimetrically by measuring the amount of reducing sugars released using dinitrosalicylic acid (DNSA) [29]. Tubes containing 1.8 mL of substrate were placed in a thermostat at 39 °C for 5 min. One hundred microlitres of the homogenate were then added to the tubes (50 µL for xylanase), and the contents of the tubes were supplemented with 100 µL (150 µL for xylanase) of a phosphate-citrate

 doi:10.20944/preprints202307.1365.v1

buffer solution to a volume of 200 µL. The mixtures were boiled in 3 mL of DNSA (1% solution) for 5 min to stop the reaction. The blank was prepared similarly to the experimental samples using deionized water instead of the homogenate. Standards were prepared from standard solutions and a phosphate-citrate buffer with final volumes of 200 µL. All samples, standards, and enzymatic and reaction blanks were prepared in duplicate and boiled for 5 min and cooled, and 250 µL were transferred to a 96-well plate. Absorbances were spectrophotometrically measured against the reaction blank at a wavelength of 540 nm [30]. Enzymatic activity was expressed in units of specific catalytic activity (cat/g of protein). Amylase activity was determined using 0.2% (w/v) maize starch (Merck KGaA, Darmstadt, Germany) resuspended in 0.05 M phosphate-citrate buffer. Cellulase activity was determined using 1% (w/v) carboxymethyl cellulose (Merck KGaA, Darmstadt, Germany). Xylanase activity was determined using 1% (w/v) Beechwood xylan (Merck KGaA, Darmstadt, Germany) resuspended in the same phosphate-citrate buffer. Enzymatic activities were determined by measuring the amount of reducing sugars released from the samples after 15 min at 39 °C.

2.5. Chemical Analysis of Dietary Substrates

The chemical compositions, phytochemical substances, and FA profiles of the dietary substrates used in both the in vivo and in vitro experiments are presented in Table 1.

Table 1. Chemical composition, phytochemical substances and fatty acid profile of the substrates.

Substrate	Meadow hay	Concentrate	Herbmix	Chicory
Dry matter (g/kg)	884	886	902	927
Chemical composition (g/kg DM)				
NDF [1]	523	227	405	384
ADF [2]	323	135	254	292
CP [3]	144	217	194	198
N [4]	23	30	31	23
Ash	138	114	143	74
Polyphenols (mg/g DM)				
Flavonoids	ND [10]	ND	22.5	9.08
Phenolic acids	ND	ND	14.8	16.9
Alkaloids	ND	ND	2.97	ND
Coumarins	ND	ND	ND	4.71
Fatty acid profile (g/100 g FA)				
C14:0	1.09	0.98	1.38	0.97
C14:1	0.10	0.72	0.07	0.08
C16:0	24.6	17.8	26.9	21.2
C16:1	0.66	0.69	0.60	0.25
C18:0	4.14	4.96	9.79	5.86
C18:1cis9	9.36	12.7	17.1	10.5
C18:2cis9cis12	18.6	53.2	28.9	32.4
C18:3cis9cis12cis15	32.1	4.88	8.73	22.3
Other FA [5]	9.42	4.12	6.46	6.42
SFA [6]	33.0	25.4	40.8	30.0
UFA [7]	67.0	74.6	59.2	70.0
MUFA [8]	13.0	15.9	19.5	13.0
PUFA [9]	54.0	58.7	39.7	57.0
n-6	21.8	53.7	30.9	34.6
n-3	32.3	5.00	8.83	22.4
n6/n3	0.68	10.7	3.49	1.55

[1] Neutral detergent fiber; [2] acid detergent fiber; [3] crude protein; [4] nitrogen; [5] other fatty acids (C18:1cis11, C20:3n6, C22:1n9, C20:3n3, C20:4n6, C23:0, C22:2, C24:0); [6] saturated FA; [7] unsaturated FA; [8] monounsaturated FA; [9] polyunsaturated FA; [10] not determined.

 doi:10.20944/preprints202307.1365.v1

2.5.1. Diet Composition

The dietary substrates were analysed in triplicate using standard procedures [31,32] as was previously described [33]. The analyses were performed using an ANKOM 2000 Automated Fiber Analyzer (Ankom Technology, Macedon, USA) and a FLASH 4000 N/Protein Analyzer (Thermo Fisher Scientific, Cambridge, UK).

2.5.2. Phytochemical Substances

The bioactive compounds of Herbmix and the chicory were quantified using ultrahigh-resolution mass spectrometry on a Dionex UltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector connected to a Compact high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) as previously described [20,34].

2.5.3. FA Profiles

The FA concentrations were determined using standard protocols [35]. FAs were identified and quantified based on their peaks and retention times by comparing FA sample targets with appropriate FA methyl ester standards (Supelco 37 Component FAME Mix) (Sigma-Aldrich, Saint-Louis, Missouri, USA). The concentrations of conjugated linoleic acid (CLA) were determined using a CLA standard (a mixture of cis 9, trans 11 and trans 10, cis 12-octadecadienoic acid methyl esters; Sigma-Aldrich) on a Galaxie Work Station 10.1 (Varian, Walnut Creek, USA).

2.6. Calculations and Statistical Analysis

Methane production and hydrogen production and use were estimated based on stoichiometric calculations [36]. Methane concentration was calculated by measuring the molar proportions of the SCFAs 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. Fermentation parameters and microbial populations in vivo were analysed using an unpaired *t*-test (GraphPad Prism 9.2.0 (332) 2021; GraphPad Software, Inc., San Diego, USA). Fermentation parameters and protozoan populations in vitro were analysed using a two-way ANOVA in a 3 × 2 × 3 factorial arrangement in a completely randomised design. The model included effects for substrate, inoculum, and interaction as fixed factors, and each consecutive run was considered as a random factor. Individual differences were determined using Tukey's multiple comparison *post hoc* tests and were considered significant at $p < 0.05$.

3. Results

3.1. Parasitological Status and Body Weight

Total lamb body weight (BW) did not differ significantly between groups ($p > 0.05$) (Table 2). The INF lambs were infection positive, with a mean EPG of 18 200 ± 5225.

Table 2. Body weight and EPG of lambs (n = 6).

Parameter	Day	Control	Infection	*p*
BW (kg)	0	18.6 ± 3.94 [1]	19.8 ± 1.40	0.498
	50	25.1 ± 2.41	23.3 ± 1.89	0.181
EPG (mean counts)	48	-	18200 ± 5225	-

[1] Mean ± standard deviation (SD); BW, body weight; EPG, eggs per gramme of feces.

3.2. Ruminal Fermentation In Vivo

Infection significantly affected ammonia-N and methane concentrations in the lambs, with both higher for INF than CON (Table 3). The concentrations of *n*-butyrate ($p = 0.010$), *iso*-butyrate ($p =$

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 July 2023 doi:10.20944/preprints202307.1365.v1

0.018), and *iso*-valerate were significantly lower, but the acetate:propionate ratio (p = 0.022) was significantly higher, for INF than CON.

Table 3. Fermentation parameters in lambs (n = 6).

Parameter	Control	Infection	p
pH	6.72 ± 0.541 [1]	6.84 ± 0.540	0.709
Ammonia -N (mg/L)	66.2 ± 33.7	130 ± 37.4	0.011
CH_4 (mmoL) [1]	0.40 ± 0.010	0.49 ± 0.012	< 0.001
CH_4 production (mmoL) [2]	17.3 ± 0.527	18.1 ± 0.523	0.021
H_2 production (mmoL) [2]	185 ± 32.1	192 ± 33.0	0.729
H_2 utilization (mmoL) [2]	127 ± 28.5	135 ± 25.1	0.646
H_2 recovery (%) [2]	69 ± 8.2	70 ± 7.1	0.790
Total SCFA (mmoL/L)	59.0 ± 11.82	68.0 ± 27.7	0.481
Acetate (mol%)	69.3 ± 7.2	75.1 ± 10.4	0.287
Propionate (mol%)	16.0 ± 2.43	14.1 ± 2.50	0.207
n-Butyrate (mol%)	11.1 ± 0.69	9.05 ± 0.85	0.010
iso-Butyrate (mol%)	1.10 ± 0.1	0.24 ± 0.02	0.018
n-Valerate (mol%)	1.40 ± 0.55	1.30 ± 0.54	0.757
iso-Valerate (mol%)	0.24 ± 0.02	0.20 ± 0.05	0.003
Caproate (mol%)	0.15 ± 0.01	0.15 ± 0.02	0.157
Acetate: Propionate	4.33 ± 0.63	5.33 ± 0.60	0.022

[1] Mean ± SD; SCFA, total short-chain fatty acids; wRC, count per gramme of wet ruminal content; [2] CH_4: methane estimation [36].

3.3. Ruminal Microbiota and Specific Enzymatic Activities

The populations of protozoa (p < 0.001), bacteria (p < 0.001), total *Archaea* (p < 0.001), *Methanobacteriales* (p < 0.001), and *Methanomicrobiales* (p = 0.002) were significantly higher for INF than CON (Table 4). The specific enzymatic activity of amylase (p = 0.015) was significantly lower for INF than CON.

Table 4. Ruminal microbiota and specific enzymatic activities in lambs (n = 6).

Parameter.	Control	Infection	p
Total protozoa (10^5/g wRC)	5.11 ± 0.45 [1]	6.90 ± 0.52	< 0.001
Total bacteria (10^8/mL)	3.20 ± 0.676	4.27 ± 0.761	< 0.001
Archaea (10^7/mL)	7.35 ± 0.275	8.16 ± 0.280	< 0.001
Methanobacteriales (10^7/mL)	2.07 ± 0.122	3.21 ± 0.118	< 0.001
Methanomicrobiales (10^7/mL)	2.87 ± 0.760	3.94 ± 0.757	0.002
Specific enzymatic activities (μkat/g protein)			
Amylase	2.81 ± 0.598	1.74 ± 0.659	0.015
Cellulase	1.87 ± 0.834	1.44 ± 0.584	0.325
Xylanase	142.9 ± 23.8	152.2 ± 44.2	0.660

[1] Mean ± SD.

3.4. Ruminal Fermentation In Vitro

The effects of the inocula and substrates on the parameters of ruminal fermentation in vitro are presented in Table 5. The substrates significantly affected the concentrations of *n*-butyrate (p = 0.048), *iso*-butyrate (p < 0.001), *n*-valerate (p < 0.001), *iso*-valerate (p < 0.001), ammonia-N (p = 0.021), total gas (p = 0.001), and methane (p < 0.001). The inocula significantly affected the concentrations of *n*-butyrate (p = 0.002), ammonia-N (p = 0.004), and methane (p < 0.001). The inocula also significantly affected the total number of protozoa (p = 0.029). The inoculum × substrate interaction significantly affected only the concentrations of *n*-butyrate (p = 0.002), *n*-valerate (p = 0.013), and methane (p < 0.001).

 doi:10.20944/preprints202307.1365.v1

Table 5. Fermentation parameters after 24 h of in vitro incubation (n = 9).

Substrate	Inoculum	SCFAs	A	P	n-B	i-B	n-V	i-V	A:P	pH	NH_3-N	TGP	CH_4	PROT
		(mM)	(Mol%)								(mg/L)	(mL/g DM)	(mM)	(10^3/mL)
Meadow hay	CON	54.6	70.1	25.2	12.9	0.77	1.73	0.57	2.78	7.12	85	160	1.70	48.7
	INF	52.7	63.8	24.9	9.21	0.82	1.43	0.50	2.56	7.02	126	180	2.67	71.8
Herbmix	CON	53.9	67.1	26.1	10.4	0.99	1.79	0.77	2.57	7.01	92	220	1.55	57.8
	INF	53.6	69.0	25.7	9.22	0.95	1.96	0.71	2.69	7.15	143	232	3.49	68.7
Chicory	CON	54.5	65.6	24.8	13.0	1.17	1.54	0.93	2.65	7.20	87	150	1.86	63.0
	INF	52.0	64.0	23.9	12.0	1.16	1.51	0.89	2.68	7.15	157	153	1.64	65.9
Significance of the effects:														
Substrate		0.999	0.934	0.815	0.048	0.001	0.001	0.001	0.991	0.834	0.021	0.001	0.001	0.814
Inoculum		0.864	0.827	0.788	0.002	0.987	0.413	0.114	0.929	0.985	0.004	0.068	0.001	0.029
Substrate × Inoculum		0.995	0.934	0.991	0.002	0.839	0.013	0.939	0.858	0.834	0.693	0.544	0.001	0.327

SCFAs, total short-chain fatty acids; A, acetate; P, propionate; i-B, *iso*-butyrate; n-B, *n*-butyrate; i-V, *iso*-valerate; n-V, *n*-valerate; TGP, total gas production; PROT, protozoa; SEM, standard error of means.

4. Discussion

Many studies have confirmed the negative impact of GIN infections on sheep performance [37–39]. GIN infections can reduce the voluntary feed intake in ruminants, even in the absence of any clinical disease [40]. The results of a meta-analysis indicated that weight gain in animals infected with *H. contortus* was 77% of the gain in parasite-free animals [39]. We thus also expected worse general health conditions in the infected lambs, manifested by lower BWs 50 d *post*-infection. Our experiment, however, did not confirm the effect of infection on BW, probably because feeding the lambs with quality forage ensured that the lambs were free from adverse effects on animal performance. The lambs maintained good nutritional status (nutritional management) with the forage within medium- to high-quality standards [41], which was evident from the optimal chemical composition of the dietary substrates (Table 1). The ability of ruminants to resist the negative impact of GIN infections when maintaining good nutritional status (nutritional management) has previously been reported [42,43].

Changes in the ruminal ammonia-N and methane concentrations in INF may have been associated with alteration of the ruminal microbiota. The optimal level of ammonia-N in the rumen (20-100 mg/l; [44]) was exceeded in our experiment in INF. Ruminal ammonia-N is normally the most abundant nitrogenous compound needed for microbial growth; its increase in the rumens of the infected lambs may have been due to the lower consumption of ammonia-N by microorganisms. These microorganisms have access to a readily available source of energy, increasing microbial protein synthesis or decreasing the use of amino acids as a microbial energy source [45]. A large increase in the amount of additional endogenous nitrogen entering the duodenum in sheep infected with *H. contortus* would probably lead to the loss of amino acids, because the reabsorbed N not from ammonia would likely be used inefficiently [46]. The high ammonia-N concentrations would consequently inhibit acetoclastic methanogens much more than hydrogenotrophic methanogens, so methane would mainly be formed by hydrogen-using methanogens [47,48]. Acetate oxidation associated with hydrogenotrophic methanogenesis is probably the dominant metabolic pathway for methane formation [49].

Infection with the GIN *H. contortus* alters the composition and diversity of the microbial community, which facilitates bacterial survival and reproduction. An increase in the abundance of bacterial genera associated with methanogenesis, and other ruminal microorganisms involved in microbial homeostasis, may also be affected by infection [50,51]. The ruminal microbiome may therefore be affected by decreasing butyrate and increasing ammonia-N concentrations, which would affect microorganisms and the genes controlling the metabolic pathway involved in microbial homeostasis. Changes in the ruminal microbiota, including ciliates that are responsible for methanogenesis in GIN-infected animals and that determine how hydrogen is used in the rumen, remain poorly understood. The intake and composition of feed and the ruminal microbiome, however, are the primary factors influencing enteric methane emissions.

 doi:10.20944/preprints202307.1365.v1

Concentrations of branched-chain FAs (BCFAs) can also be affected in vivo. BCFAs are formed in the rumen by the deamination of amino acids, and their concentrations depend on the ruminal degradation of dietary proteins [52]. The relative proportion of proteins in the abomasum obtained from feed and microbial proteins synthesised in the rumen, however, depends on the physiological status of lambs and on diet composition. GIN infection can therefore probably also affect the specific activities of enzymes of the ruminal microorganisms, especially amylase, which is associated with the particulate fraction [53]. Diet in our study, however, may also have contributed to the increase in amylase activity in the ruminal microorganisms in CON.

Dietary substrates containing Herbmix (a mixture of mallow, chamomile, fumitory, and wormwood [34] or chicory [54] have the potential to reduce enteric methane and ammonia-N concentrations in vitro. Our study found differences between the in vitro fermentation of the same substrates using inocula from GIN-infected and uninfected animals. The INF inocula had lower butyrate and higher ammonia-N and methane concentrations. Ammonia-N concentration was correlated with BCFA concentration, and some fermentation parameters (total gas production, SCFA concentrations, and protozoan activity) differed significantly in the resistance of substrates to degradation in the ruminal fluid, consistent with previous results [55].

The increase in BCFA concentration in vitro was probably due to the high protein contents of Herbmix and chicory [56,57], so cellulolytic microorganisms would primarily use BCFA as the main source of carbon chains for growth. Diets with a high chicory content (≥ 70% DM) can directly affect GIN parasitism [13], but some studies have also described indirect anthelmintic effects of chicory and herbal mixtures on local abomasal immunities [14,58,59]. Our in vitro fermentation with chicory also provided promising results in mitigating methane production, and chicory likely modulated the ruminal fermentation and microbiota of the GIN-infected lambs. Chicory was a source of coumarins in our experiment, and coumarin-rich raw materials can inhibit acetogenesis and acetoclastic methanogenesis when the microbiome is not adapted to coumarins [60]. Our previous results indicated that fermentation in the rumen (in vivo and in vitro) could clearly be modified by supplementing medicinal plants, without adverse effects on the parameters of fermentation in the rumen [28,33].

The high potential of tannins and flavonoids to reduce methane and ammonia-N concentrations during ruminal fermentation in vitro has also been previously described [61–63]. These bioactive compounds probably have similar mechanisms of action that also affect GIN larvae [64]. Chicory bioactive compounds may have the potential to inhibit methane production in ruminal fermentation in GIN-infected animals without adversely affecting fermentation. We could not confirm this inhibition for infected animals fed Herbmix. Antimicrobial activity varies amongst flavonoids [65], with some flavonoids enhancing the efficiency of fermentation by improving propionate production to the detriment of acetate production and reducing the hydrogenotrophic methanogenic communities of *Archaea* [66]. Our in vitro results are consistent with either the direct effects of plant bioactive compounds on methanogens or an indirect effect to reduce the production of hydrogen as a substrate for microorganisms [67]. Variations in the ruminal microbiome, fermentation kinetics, and the responses and adaptations to anti-methanogenic inhibitors and dietary substrates, however, may be important factors influencing the efficacy of bioactive compounds [68]. The effectiveness of bioactive substances, though, can vary considerably depending on the type, source, molecular weight, and dose in the diet [69].

5. Conclusions

Our results provide novel insights into the benefits of bioactive plants used to reduce the negative effects of infection with gastrointestinal nematodes, i.e., the microbiota and patterns of ruminal fermentation in lambs. An association between GIN infection by *H. contortus* and increased methanogenesis was observed. Chicory has promise for mitigating ruminal methane production and may modulate the ruminal fermentation and microbiota of GIN-infected lambs. The mechanisms of, and contributions of Herbmix, chicory, and gastrointestinal nematodes to, the ruminal microbiome of lambs, however, remain undefined. More in vivo studies are thus needed.

Author Contributions: Conceptualization, K.M. and Z.V.; methodology, K.M.; software, D.P.; validation, M.K. (Michaela Komáromyová); D.B. and M.K. (Martyna Kozłowska); formal analysis, S.S.; investigation, K.M.; resources, K.M.; data curation, A.C.; writing—original draft preparation, Z.V.; writing—review and editing, Z.V. and A.C.; supervision, Z.V.; project administration, M.V.; funding acquisition, M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by funds from the Slovak Research and Development Agency (www.apvv.sk accessed on 1 July 2019) (APVV 18-0131) and the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences (https://www.minedu.sk/vedecka-grantova-agentura-msvvas-sr-a-sav-vega/ accessed on 4 April 2021) (VEGA 2/0008/21). This research was also partially funded by the Faculty of Veterinary Medicine and Animal Science, Poznań University of Life Sciences, Poland, by the Department of Animal Nutrition (no. 506.533.04.00).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences on 16 February 2022 (protocol code 2022/09).

Data Availability Statement: Data are available upon reasonable request to the corresponding author.

Acknowledgments: In The authors are grateful to Valéria Venglovská and Peter Jerga for their technical support.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Charlier, J.; Rinaldi, L.; Musella, V.; Ploeger, H.W.; Chartier, C.; Rose Vineer, H.; Hinney, B.; von Samson-Himmelstjerna, G.; Băcescu, B.; Mickiewicz, M.; Mateus, T.L.; Martinez-Valladares, M.; Quealy, S.; Azaizeh, H.; Sekovska, B.; Akkari, H.; Petkevicius, S.; Hektoen, L.; Höglund, J.; Morgan, E.R.; Bartley, D.J.; Claerebout, E. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. *Prev. Vet. Med.* **2020**, *182*, 105103. https://doi.org/10.1016/j.prevetmed.2020.105103
2. Houdijk, J.G.M.; Tolkamp, B.J.; Rooke, J.A.; Hutchings, M.R. Animal health and greenhouse gas intensity: the paradox of periparturient parasitism. *Int. J. Parasitol.* **2017**, *47*, 633–641. https://doi.org/10.1016/j.ijpara.2017.03.006
3. Fox, N.J.; Smith, L.A.; Houdijk, J.G.M.; Athanasiadou, S.; Hutchings, M.R. Ubiquitous parasites drive a 33% increase in methane yield from livestock. *Int. J. Parasitol.* **2018**, *48*, 1017–1021. https://doi.org/10.1016/j.ijpara.2018.06.001
4. Lima, P.M.T.; Crouzoulon, P.; Sanches, T.P.; Zabré, G.; Kabore, A.; Niderkorn, V.; Hoste, H.; Amarante, A.F.T.D.; Costa-Júnior, L.M.; Abdalla, A.L.; Louvandini, H. Effects of *Acacia mearnsii* supplementation on nutrition, parasitological, blood parameters and methane emissions in Santa Inês sheep infected with *Trichostrongylus colubriformis* and *Haemonchus contortus*. *Exp. Parasitol.* **2019**, *207*, 107777. https://doi.org/10.1016/j.exppara.2019.107777
5. El-Ashram, S.; Al Nasr, I.; Abouhajer, F.; El-Kemary, M.; Huang, G.; Dinçel, G.; Mehmood, R.; Hu, M.; Suo, X. Microbial community and ovine host response varies with early and late stages of *Haemonchus contortus* infection. *Vet. Res. Commun.* **2017**, *41*, 263–277. https://doi.org/10.1007/s11259-017-9698-5
6. Mizrahi, I.; Jami, E. Review: the compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal* **2018**, *12*, 220–232. https://doi.org/10.1017/S1751731118001957
7. Clemmons, B.A.; Voy, B.H.; Myer, P.R. Altering the gut microbiome of cattle: considerations of host-microbiome interactions for persistent microbiome manipulation. *Microb. Ecol.* **2019**, *77*, 523–536. https://doi.org/10.1007/s00248-018-1234-9
8. Li, R. W.; Li, W.; Sun, J.; Yu, P.; Baldwin, R.L.; Urban, J.F. The effect of helminth infection on the microbial composition and structure of the caprine abomasal microbiome. *Sci. Rep.* **2016**, *6*, 20606. https://doi.org/10.1038/srep20606
9. Zaiss, M.M.; Harris, N.L. Interactions between the intestinal microbiome and helminth parasites. *Parasite Immunol.* **2016**, *38*, 5–11. https://doi.org/10.1111/pim.12274
10. Gruninger, R.J.; Ribeiro, G.O.; Cameron, A.; McAllister, T.A. Invited review: Application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal* **2019**, *13*, 1843–1854. https://doi.org/10.1017/S1751731119000752
11. Spiegler, V.; Liebau, E.; Hensel, A. Medicinal plant extracts and plant-derived polyphenols with anthelmintic activity against intestinal nematodes. *Nat. Prod. Rep.* **2017**, *34*, 627–643. https://doi.org/10.1039/c6np00126b

12. Valenzuela-Grijalva, N.V.; Pinelli-Saavedra, A.; Muhlia-Almazan, A.; Domínguez-Díaz, D.; González-Ríos, H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J. Anim. Sci. Technol.* **2017**, *59*, 8. https://doi.org/10.1186/s40781-017-0133-9
13. Peña-Espinoza, M.; Valente, A.H.; Thamsborg, S.M.; Simonsen, H.T.; Boas, U.; Enemark, H.L.; López-Muñoz, R.; Williams, A.R. Antiparasitic activity of chicory (*Cichorium intybus*) and its natural bioactive compounds in livestock: a review. *Parasit. Vectors.* **2018**, *11*, 475. https://doi.org/10.1186/s13071-018-3012-4
14. Mravčáková, D.; Komáromyová, M.; Babják, M.; Urda Dolinská, M.; Königová, A.; Petrič, D.; Čobanová, K.; Ślusarczyk, S.; Cieslak, A.; Várady, M.; Váradyová, Z. Anthelmintic activity of wormwood (*Artemisia absinthium* L.) and mallow (*Malva sylvestris* L.) against *Haemonchus contortus* in sheep. *Animals* **2020**, *10*, 219. http://dx.doi.org/10.3390/ani10020219
15. Mravčáková, D.; Sobczak-Filipiak, M.; Váradyová, Z.; Kucková, K.; Čobanová, K.; Maršík, P.; Tauchen, J.; Vadlejch, J.; Mickiewicz, M.; Kaba, J.; Várady, M. Effect of *Artemisia absinthium* and *Malva sylvestris* on antioxidant parameters and abomasal histopathology in lambs experimentally infected with *Haemonchus contortus*. *Animals* **2021**, *11*, 462. https://doi.org/10.3390/ani11020462
16. Kim, J.H.; Kim, C.H.; Ko, Y.D. Influence of dietary addition of dried wormwood (*Artemisia* sp.) on the performance and carcass characteristics of Hanwoo steers and the nutrient digestibility of sheep. *Asian-australas. J. Anim. Sci.* **2002**, *15*, 390–395.
17. Mayer, M.; Vogl, C.R.; Amorena, M.; Hamburger, M.; Walkenhorst, M. Treatment of organic livestock with medicinal plants: a systematic review of European ethnoveterinary research. *Forsch. Komplementmed.* **2014**, *21*, 375–386. https://doi.org/10.1159/000370216
18. Sun, X.; Cheng, L.; Jonker, A.; Munidasa, S.; Pacheco, D. A review: plant carbohydrate types-the potential impact on ruminant methane emissions. *Front. Vet. Sci.* **2022**, *9*, 880115. https://doi.org/10.3389/fvets.2022.880115
19. Váradyová, Z.; Pisarčíková, J.; Babják, M.; Hodges, A.; Mravčáková, D.; Kišidayová, S.; Königová, A.; Vadlejch, J.; Várady, M. Ovicidal and larvicidal activity of extracts from medicinal plants against *Haemonchus contortus*. *Exp. Parasitol.* **2018,** *195*, 71–77. https://doi.org/10.1016/j.exppara.2018.10.009
20. Váradyová, Z.; Mravčáková, D.; Babják, M.; Bryszak, M.; Grešáková, Ľ.; Čobanová, K.; Kišidayová, S.; Plachá, I.; Königová, A.; Cieslak, A.; Slusarczyk, S.; Pecio, L.; Kowalczyk, M.; Várady, M. Effects of herbal nutraceuticals and/or zinc against *Haemonchus contortus* in lambs experimentally infected. *BMC Vet. Res.* **2018**, *14*, 78. https://doi.org/10.1186/s12917-018-1405-4
21. Coles, G.C.; Bauer, C.; Borgsteede, F.H.M.; Geerts, S.; Klei, T.R.; Taylor, M.A.; Waller, P.J. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasitol.* **1992,** 44, 35–44. https://doi.org/10.1016/0304-4017(92)90141-u
22. European Commission (EC). Council Regulation (EC) 1099/2009 of 24 September 2009 on the Protection of Animals at the Time of Killing. Available online: https://eur-lex.europa.eu/legal-content /EN/TXT/?uri=celex:32009R1099 (accessed on 18 November 2009).
23. McDougall, E.I. Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochem. J.* **1948**, *43*, 99–109.
24. Váradyová, Z.; Baran, M.; Zeleňák, I. Comparison of two in vitro fermentation gas production methods using both rumen fluid and faecal inoculum from sheep. *Anim. Feed Sci. Technol.* **2005**, *123–124*, 81–94. https://doi:10.1016/j.anifeedsci.2005.04.030
25. Váradyová, Z.; Kišidayová, S.; Čobanová, K.; Grešáková, Ľ.; Babják, M.; Königová, A.; Urda Dolinská, M.; Várady, M. The impact of a mixture of medicinal herbs on ruminal fermentation, parasitological status and hematological parameters of the lambs experimentally infected with *Haemonchus contortus*. *Small Rumin. Res.* **2017**, *151*, 124–132. https://doi.org/10.1016/j.smallrumres.2017.04.023
26. Broderick, G.A.; Kang, J.H. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. *J. Dairy Sci.* **1980**, *63*, 64–75. https://doi.org/10.3168/jds.S0022-0302(80)82888-8
27. Williams, A.G.; Coleman, G.S. *The Rumen Protozoa*, 1st ed.; Springer Verlag: New York, NY, 1992; pp. 1–425.
28. Szulc, P.; Mravčáková, D.; Szumacher-Strabel, M.; Váradyová, Z.; Várady, M.; Čobanová, K.; Syahrulawal, L.; Patra, A.K.; Cieslak, A. Ruminal fermentation, microbial population and lipid metabolism in gastrointestinal nematode-infected lambs fed a diet supplemented with herbal mixtures. *PLoS ONE* **2020**, *15*, e0231516. https://doi.org/doi.org/10.1371/journal.pone.0231516
29. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959,** *31*, 426–428. https://doi.org/10.1021/ac60147a030
30. Bailey, M.J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257–270. https://doi.org/10.1016/0168-1656(92)90074-J
31. Horwitz, W. *Official Methods of AOAC International*, 17th ed.; Association of Official Analytical Chemists (AOAC) International: Gaithersburg, MD, USA; Washington, DC, USA, 2000.

32. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
33. Váradyová, Z.; Mravčáková, D.; Holodová, M.; Grešáková, Ľ.; Pisarčíková, J.; Barszcz, M.; Taciak, M.; Tuśnio, A.; Kišidayová, S.; Čobanová, K. Modulation of ruminal and intestinal fermentation by medicinal plants and zinc from different sources. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 1131–1145. https://doi.org/10.1111/jpn.12940
34. Petrič, D.; Mravčáková, D.; Kucková, K.; Čobanová, K.; Kišidayová, S.; Cieslak, A.; Slusarczyk, S.; Váradyová, Z. Effect of dry medicinal plants (wormwood, chamomile, fumitory and mallow) on in vitro ruminal antioxidant capacity and fermentation patterns of sheep. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 1219–1232. https://doi.org/10.1111/jpn.13349
35. Cieslak, A.; Stanisz, M.; Wojtowski, J.; Pers-Kamczyc, E.; Szczechowiak, J.; El-Sherbiny, M.; Szumacher-Strabel, M. *Camelina sativa* affects the fatty acid contents in *M. longissimus* muscle of lambs. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 1258–1265. https://doi.org/10.1002/ejlt.201200119
36. Wolin, M.J. A Theoretical Rumen Fermentation Balance. *J. Dairy Sci.* **1960,** *43*, 1452–1459. https://doi.org/10.3168/jds.S0022-0302(60)90348-9
37. Sutherland, I.; Scott, I. *Gastrointestinal nematodes of sheep and cattle: biology and control*; Wiley-Blackwell: West Sussex, UK, 2010; pp. 1–242.
38. Cornelius, M.P.; Jacobson, C.; Besier, R.B. Body condition score as a selection tool for targeted selective treatment-based nematode control strategies in Merino ewes. *Vet. Parasitol.* **2014**, *206*, 173–181. https://doi.org/10.1016/j.vetpar.2014.10.031
39. Mavrot, F.; Hertzberg, H.; Torgerson, P. Effect of gastro-intestinal nematode infection on sheep performance: A systematic review and meta-analysis. *Parasit. Vectors* **2015**, *8*, 557. https://doi.org/10.1186/s13071-015-1164-z
40. Forbes, A.B.; Huckle, C.A.; Gibb, M.J.; Rook, A.J.; Nuthall, R. Evaluation of the effects of nematode parasitism on grazing behaviour, herbage intake and growth in young grazing cattle. *Vet. Parasitol.* **2020**, *90*, 111–118. https://doi.org/10.1016/s0304-4017(00)00218-1
41. Ball, D.M.; Collins, M.; Lacefield, G.D.; Martin, N.P.; Mertens, D.A.; Olson, K.E.; Putnam, D.H.; Undersander, D.J.; Wolf, M.W. *Understanding Forage Quality*. American Farm Bureau Federation Publication 1-01, Park Ridge, IL. 2001.
42. Coop, R.L.; Holmes, P.H. Nutrition and parasite interaction. *Internat. J. Parasitol.* **1996**, *26*, 951–962. https://doi.org/10.1016/s0020-7519(96)80070-1
43. Steel, J.W. Effects of protein supplementation on young sheep on resistance development and resilience to parasitic nematodes. *Aust. J. Exp. Agric.* **2003**, *12*, 1469–1476. https://doi.org/10.1071/EA03004
44. Pisulewski, P.M.; Okorie, A.U.; Buttery, P.J.; Haresign, W.; Lewis, D. Ammonia concentration and protein synthesis in the rumen. *J. Sci. Food Agric.* **1981**, *32*, 759–766. https://doi.org/10.1002/jsfa.2740320803
45. Nocek, J.E.; Russell, J.B. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability for microbial synthesis and milk production. *J. Dairy Sci.* **1988**, *71*, 2070–2107. https://doi.org/10.3168/jds.S0022-0302(88)79782-9
46. Rowe, J.; Nolan, J.; De Chaneet, G.; Teleni, E.; Holmes, P. The effect of haemonchosis and blood loss into the abomasum on digestion in sheep. *Br. J. Nutr.* **1988**, *59*, 125–139. https://doi.org/10.1079/BJN19880016
47. Karakashev, D.; Batstone, D.J.; Angelidaki, I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microbiol.* **2005**, *71*(1), 331–338. https://doi.org/10.1128/AEM.71.1.331-338
48. Karakashev, D.; Batstone, D.J.; Trably, E.; Angelidaki, I. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of *Methanosaetaceae*. *Appl. Environ. Microbiol.* **2006,** *72*(7), 5138–5141. https://doi.org/10.1128/AEM.00489-06
49. Muñoz-Tamayo, R.; Popova, M.; Tillier, M.; Morgavi, D.P.; Morel, J.-P.; Fonty, G.; Morel-Desrosiers, N. Hydrogenotrophic methanogens of the mammalian gut: Functionally similar, thermodynamically different—A modelling approach. *PLoS ONE* **2019**, *14*(12), e0226243. https://doi.org/10.1371/journal.pone.0226243
50. Corrêa, P.S.; Mendes, L.W.; Lemos, L.N.; Crouzoulon, P.; Niderkorn, V.; Hoste, H.; Costa-Júnior, L.M.; Tsai, S.M.; Faciola, A.P.; Abdalla, A.L.; Louvandini, H. Tannin supplementation modulates the composition and function of ruminal microbiome in lambs infected with gastrointestinal nematodes. *FEMS Microbiol. Ecol.* **2020**, *96*, fiaa024. https://doi.org/10.1093/femsec/fiaa024
51. Corrêa, P.S.; Mendes, L.W.; Lemos, L.N.; Sampaio, A.C.K.; Issakowicz, J.; McManus, C.M.; Tsai, S.M.; Faciola, A.P.; Abdalla, A.L.; Louvandini, H. The effect of *Haemonchus contortus* and *Trichostrongylus colubriforms* infection on the ruminal microbiome of lambs. *Exp. Parasitol.* **2021**, *231*, 108175. https://doi.org/10.1016/j.exppara.2021.108175.
52. Allison, M.J. Production of branched-chain volatile fatty acids by certain anaerobic bacteria. *Appl. Environ. Microbiol.* **1978**, *35*, 872–877. https://doi.org/10.1128/aem.35.5.872-877.1978

53. Raffrenato, E.; Badenhorst, M.J.; Shipandeni, M.N.T.; van Zyl, W.H. Rumen fluid handling affects measurements of its enzymatic activity and in vitro digestibility. *Anim. Feed Sci. Technol.* **2021**, *280*, 115060. https://doi.org/10.1016/j.anifeedsci.2021.115060
54. Niderkorn, V.; Martin, C.; Bernard, M.; Le Morvan, A.; Rochette, Y.; Baumont, R. Effect of increasing the proportion of chicory in forage-based diets on intake and digestion by sheep. *Animal* **2019**, *13*(4), 718–726. https://doi.org/10.1017/S1751731118002185
55. Apajalahti, J.; Vienola, K.; Raatikainen, K.; Holder, V.; Moran, C.A. Conversion of branched-chain amino acids to corresponding isoacids - An in vitro tool for estimating ruminal protein degradability. *Front. Vet. Sci.* **2019**, *6*, 311. https://doi.org/10.3389/fvets.2019.00311
56. Nwafor, I.C.; Shale, K.; Achilonu, M.C. Chemical composition and nutritive benefits of chicory (*Cichorium intybus*) as an ideal complementary and/or alternative livestock feed supplement. *Sci. World J.* **2017**, *2017*, 7343928. https://doi.org/10.1155/2017/7343928
57. Petrič, D.; Mravčáková, D.; Kucková, K.; Kišidayová, S.; Cieslak, A.; Szumacher-Strabel, M.; Huang, H.; Kolodziejski, P.; Lukomska, A.; Slusarczyk, S.; Čobanová, K.; Váradyová, Z. Impact of zinc and/or herbal mixture on ruminal fermentation, microbiota, and histopathology in lambs. *Front. Vet. Sci.* **2021,** *8*, 630971. https://doi.org/10.3389/fvets.2021.630971
58. Tzamaloukas, O.; Athanasiadou, S.; Kyriazakis, I.; Huntley, J.F.; Jackson, F. The effect of chicory (*Cichorium intybus*) and sulla (*Hedysarum coronarium*) on larval development and mucosal cell responses of growing lambs challenged with *Teladorsagia circumcincta*. *Parasitology* **2006**, *132*, 419–426. https://doi.org/10.1017/S0031182005009194
59. Athanasiadou, S.; Gray, D.; Younie, D.; Tzamaloukas, O.; Jackson, F.; Kyriazakis, I. The use of chicory for parasite control in organic ewes and their lambs. *Parasitology* **2007**, *134*, 299–307. https://doi.org/10.1017/S0031182006001363
60. Popp, D.; Plugge, C.M.; Kleinsteuber, S.; Harms, H.; Sträuber, H. Inhibitory effect of coumarin on syntrophic fatty acid-oxidizing and methanogenic cultures and biogas reactor microbiomes. *Appl. Environ. Microbiol.* **2017**, *83*(13), e00438-17. https://doi.org/10.1128/AEM.00438-17
61. Bhatta, R.; Baruah, L.; Saravanan, M.; Suresh, K.P.; Sampath, K.T. Effect of medicinal and aromatic plants on rumen fermentation, protozoa population and methanogenesis in vitro. *J. Anim. Physiol. Anim. Nutr.* **2013**, *97*, 446–456. https://doi.org/10.1111/j.1439-0396.2012.01285.x.
62. Cieslak, A.; Zmora, P.; Matkowski, A.; Nawrot-Hadzik, I.; Pers-Kamczuc, E.; El Sherbiny, M.; Bryszak, M.; Szumacher-Strabel, M. Tannins from *Sanguisorba officinalis* affect in vitro rumen methane production and fermentation. *J. Anim. Plant Sci.* **2016**, *26*, 54–62.
63. Sinz, S.; Kunz, C.; Liesegang, A.; Braun, U.; Marquardt, S.; Soliva, C.R.; Kreuzer, M. In vitro bioactivity of various pure flavonoids in ruminal fermentation, with special reference to methane formation. *Czech J. Anim. Sci.* **2018**, *63*, 293–304. https://doi.org/10.17221/118/2017-CJAS
64. Barrau, E.; Fabre, N.; Fouraste, I.; Hoste, H. Effect of bioactive compounds from sainfoin (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. *Parasitology* **2005**, *131*, 531–538. https://doi.org/10.1017/S0031182005008024
65. Oskoueian, E.; Abdullah, N.; Oskoueian, A. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Res. Int.* **2013**, *2013*, 349129. http://dx.doi.org/10.1155/2013/349129
66. Seradj, A.R.; Abecia, L.; Crespo, J.; Villalba, D.; Fondevila, M.; Balcells, J. The effect of Bioflavex® and its pure flavonoid components on in vitro fermentation parameters and methane production in rumen fluid from steers given high concentrate diets. *Anim. Feed Sci. Technol.* **2014**, *197*, 85–91. https://doi.org/10.1016/j.anifeedsci.2014.08.013
67. Tavendale, M.H.; Meagher, L.P.; Pacheco, D.; Walker, N.; Attwood, G.T.; Sivakumaran, S. Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Anim. Feed Sci. Technol.* **2005**, *123–124*, 403–419. https://doi.org/10.1016/j.anifeedsci.2005.04.037
68. Patra, A.; Park, T.; Kim, M.; Yu, Z. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *J. Anim. Sci. Biotechnol.* **2017**, *8*,13. https://doi.org/10.1186/s40104-017-0145-9
69. Joch, M.; Mrázek, J.; Skřivanová, E.; Čermák, L.; Marounek, M. Effects of pure plant secondary metabolites on methane production, rumen fermentation and rumen bacteria populations in vitro. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 869–881. https://doi.org/10.1111/jpn.12910