

Article

Growth Performance and Ruminal Fermentation in Lambs with Endoparasites and In Vitro Effect of Medicinal Plants

Klára Mikulová ^{1,2}, Daniel Petrič ¹, Michaela Komáromyová ³, Dominika Battányi 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.); mravcakov@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, Wrocław Medical University, Borowska 211, 50-556 Wrocław, Poland; sylwester.slusarczyk@umed.wroc.pl

* Correspondence: varadyz@saske.sk; Tel.: +421-55-792-2972

Abstract: We investigated growth performance and ruminal fermentation associated with gastrointestinal nematode (GIN) *Haemonchus contortus* in lambs and in vitro ruminal fermentation of mallow, chamomile, fumitory, wormwood (Herbmix), and chicory using inoculum from GIN-infected lambs. Twelve lambs were equally divided into two groups: uninfected animals (CON) and animals infected (INF) with approximately 5000 third-stage larvae derived from the MHCo1 strain of GIN *H. contortus*. 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 for 24 h in vitro with 0.25 g meadow hay, Herbmix, and chicory using an in vitro gas production technique. Daily weight gain was relatively lower in the INF than the CON group, but not significantly (72.6 vs. 130.1 g/day). The ruminal populations of protozoa, bacteria, total *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* were significantly higher in the INF than in the CON group. The substrates affected the concentrations of *n*-butyrate, *iso*-butyrate, *n*-valerate, *iso*-valerate, ammonia-N, total gas, and methane ($p < 0.001$) in vitro. GIN infection affected fermentation and microbial population in the rumens of the lambs, and chicory was a promising substrate to modulate ruminal fermentation in vitro.

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



Citation: Mikulová, K.; Petrič, D.; Komáromyová, M.; Battányi, D.; Kozłowska, M.; Cieslak, A.; Ślusarczyk, S.; Várady, M.; Váradyová, Z. Growth Performance and Ruminal Fermentation in Lambs with Endoparasites and In Vitro Effect of Medicinal Plants. *Agriculture* **2023**, *13*, 1826. <https://doi.org/10.3390/agriculture13091826>

Received: 24 August 2023

Revised: 14 September 2023

Accepted: 15 September 2023

Published: 18 September 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

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 7 to 50 d post-infection has been confirmed [5].

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 SCFAs 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, and their diversity, synergy, and various combinations may contribute to pharmacological efficacy in GIN infections [11,12].

Based on previous studies [13–15], we hypothesized that the traditional medicinal plants mallow, chamomile, fumitory, wormwood (Herbmix), and chicory would also contribute to desired changes to the *in vitro* ruminal microbial fermentation 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 [16–19]. However, analyses of ruminal microbial fermentation *in vitro* are needed to identify the possible consequences of plant bioactive components used in the nutrition of parasite-laden lambs. Therefore, in this study, we performed two separate experiments (*in vivo* and *in vitro*) related to endoparasitosis in lambs. Our objectives were to (1) determine the growth performance, ruminal fermentation, and microbial population associated with GIN-infected lambs, and (2) determine the *in vitro* fermentation of Herbmix and chicory as substrates fermented with inoculum from GIN-infected lambs.

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. Experiment 1—*In Vivo*

2.2.1. 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 together 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 twelve 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 larvae derived from the MHCo1 strain of *H. contortus* susceptible to anthelmintics [20]. The nomenclature used in this manuscript is from the system used at the Moredun Research Institute, United Kingdom. A modified McMaster technique [21] with a sensitivity of 50 eggs per gram (EPG) of feces was used to detect *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].

The fresh ruminal contents were collected at a slaughterhouse before the morning feeding and immediately transported to the laboratory in a 39 °C preheated water bath.

2.2.2. 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 SCFAs and the populations of ruminal microorganisms. The concentration of ammonia-N was determined using the phenol-hypochlorite method [23]. 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 [24]. Bacteria, total *Archaea*, *Methanobacteriales*, and *Methanomicrobiales* in the ruminal contents were quantified using fluorescence in situ hybridization [25].

2.2.3. Specific Enzymatic Activities

The specific enzymatic activities of 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 homogenization and diluted in an amount of approximately 6 g with 2 mL of a phosphate–citrate buffer solution (pH 6.8) with cComplete™ mini EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The contents were homogenized in an ice bath using a 4710 series ultrasonic homogenizer (Merck KGaA, Darmstadt, Germany) at a power of 80 W pulsed in one-minute intervals (homogenization 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) [26]. Tubes containing 1.8 mL of substrate were placed in a thermostat at 39 °C for 5 min. One hundred microliters 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 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 [27]. 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.

2.3. Experiment 2—In Vitro

Ruminal contents were separately collected from the slaughtered CON and INF lambs. Ruminal contents were separately passed through four layers of gauze, mixed at a 1:2 ratio with McDougall's buffer [28], and dispensed in volumes of 35 mL into serum bottles containing 0.25 g of a substrate. 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 were obtained from a commercial source (Agrokarpoty, Plavnica, Slovak Republic) and 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 experiment was carried out using the *in vitro* gas production technique (IVGPT) on batch culture incubations of buffered ruminal fluid incubated for 24 h at 39 °C under anaerobic conditions. 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 [29]. 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 *in vitro* were analyzed on a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer, Shelton, WA, USA) [30]. Abomasal contents were collected from the slaughtered INF lambs. 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.

Experimental Design

Three replicates (three incubation serum bottles) were prepared for MH, Herbmix, or chicory, and each inoculum (CON and INF). The experiment consisted of fermentations of the three substrates in fermentations with two inocula (CON and INF) and repeated three times within three consecutive days ($n = 3 \times 3$). At the same time, three replicate bottles were also used for the blank (inoculum, no substrate).

2.4. 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)				
Neutral detergent fiber	523	227	405	384
Acid detergent fiber	323	135	254	292
Crude protein	144	217	194	198
Ash	138	114	143	74
Polyphenols (mg/g DM)				
Flavonoids	ND ²	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 ¹	9.42	4.12	6.46	6.42
Saturated FA	33.0	25.4	40.8	30.0
Unsaturated FA	67.0	74.6	59.2	70.0
Monounsaturated FA	13.0	15.9	19.5	13.0
Polyunsaturated FA	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 Other fatty acids (C18:1cis11, C20:3n6, C22:1n9, C20:3n3, C20:4n6, C23:0, C22:2, C24:0); ² not determined.

2.4.1. Diet Composition

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

2.4.2. Phytochemical Substances

The bioactive compounds in Herbmix and 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].

2.4.3. Fatty Acid Profiles

The fatty acid (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, MO, 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, CA, USA).

2.5. Statistical Analysis

Fermentation parameters and microbial populations *in vivo* were analyzed using an unpaired *t*-test (GraphPad Prism 9.2.0 (332) 2021; GraphPad Software, Inc., San Diego, CA, USA). Data on fermentation parameters and protozoan populations *in vitro* were analyzed by two-way ANOVA (GraphPad Prism 9.2.0 (332) 2021; GraphPad Software, Inc., San Diego, CA, USA). The model included effects for three substrates (MH, Herbmix, and chicory), two inocula (CON and INF), and interactions between substrates and inocula. Individual differences were determined using Tukey's multiple-comparison post-test and were considered to be significant at $p < 0.05$.

3. Results

3.1. Experiment 1

3.1.1. Parasitological Status and Body Weight

Total lamb body weight (BW), live-weight gain (LWG), and daily weight gain (DWG) did not differ significantly between groups ($p > 0.05$, Table 2). The INF lambs were infection-positive, with a mean EPG of $18\,200 \pm 5225$.

Table 2. Body weight (BW), live-weight gain (LWG), daily weight gain (DWG), and eggs per gram of feces (EPG) of lambs ($n = 6$).

Parameter	Day	Control	Infection	<i>p</i>
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
LWG (kg)	50	6.5 ± 2.93	3.5 ± 1.91	0.062
DWG (g/day)		130.1 ± 50.21	72.6 ± 38.77	0.051
EPG (mean counts)	48	-	18200 ± 5225	-

¹ Mean $\pm$ standard deviation (SD). Each animal was fed daily MH *ad libitum* and 300 g DM commercial concentrate.

3.1.2. Ruminal Fermentation *In Vivo*

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

($p = 0.018$), and *iso*-valerate ($p = 0.003$) 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	<i>p</i>
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
Total SCFA (mM/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
<i>n</i> -Butyrate (mol%)	11.1 ± 0.69	9.05 ± 0.85	0.010
<i>iso</i> -Butyrate (mol%)	1.10 ± 0.1	0.24 ± 0.02	0.018
<i>n</i> -Valerate (mol%)	1.40 ± 0.55	1.30 ± 0.54	0.757
<i>iso</i> -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; SCFAs, total short-chain fatty acids.

3.1.3. 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	<i>p</i>
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
<i>Methanobacteriales</i> (10 ⁷ /mL)	2.07 ± 0.122	3.21 ± 0.118	<0.001
<i>Methanomicrobiales</i> (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; wRC, count per gram of wet ruminal content.

3.2. Experiment 2

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 concentrations of *iso*-valerate were higher in CON groups than in INF groups. Ammonia-N concentration was higher in INF groups compared to CON groups. TGP did not differ between groups with the same substrates. The concentration of methane was highest in the INF group with Herbmix. 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 ($p < 0.001$).

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/L)	(10 ³ /mL)
Meadow hay	CON	54.6	70.1	25.2	12.9 ^b	0.77	1.73 ^c	0.57 ^b	2.78	7.12	85 ^a	160 ^{a,b}	1.70 ^b	48.7 ^a
	INF	52.7	63.8	24.9	9.21 ^a	0.82	1.43 ^a	0.50 ^a	2.56	7.02	126 ^c	180 ^b	2.67 ^d	71.8 ^e
Herbmix	CON	53.9	67.1	26.1	10.4 ^a	0.99	1.79 ^c	0.77 ^d	2.57	7.01	92 ^b	220 ^{b,c}	1.55 ^a	57.8 ^b
	INF	53.6	69.0	25.7	9.22 ^a	0.95	1.96 ^d	0.71 ^c	2.69	7.15	143 ^d	232 ^c	3.49 ^e	68.7 ^d
Chicory	CON	54.5	65.6	24.8	13.0 ^b	1.17	1.54 ^b	0.93 ^f	2.65	7.20	87 ^a	150 ^a	1.86 ^c	63.0 ^{b,c}
	INF	52.0	64.0	23.9	12.0 ^b	1.16	1.51 ^b	0.89 ^e	2.68	7.15	157 ^e	153 ^a	1.64 ^b	65.9 ^{c,d}
SEM		11.72	10.83	1.85	0.56	0.07	0.08	0.04	0.33	0.21	15.30	7.56	0.07	6.18
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. Different letters within a column indicate significant differences at $p < 0.05$.

4. Discussion

Many studies have confirmed the negative impact of GIN infections on sheep performance [36–38]. GIN infections can reduce the voluntary feed intake in ruminants, even in the absence of any clinical disease [39]. In Experiment 1, growth performance expressed as DWG was relatively lower in GIN-infected lambs compared to control, but not significantly (72.6 vs. 130.1 g/day, Table 2). 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 [38]. Most of the trials in the meta-analysis reported a negative effect of parasitism on growth performance and production, but the effect was significant in only 58.3% of the trials [38]. 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. In the present experiment, large individual differences between animals were also noted when we weighed animals. It has been documented in the past that parasitic infection with *H. contortus* can often result in a period of reduced appetite, nausea, gastroenteritis, anemia, and poor nutritional absorption, which can lead to weight loss [40]. The lambs in our experiment maintained a 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 concentration in INF in our Experiment 1 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].

In Experiment 1, concentrations of branched-chain FAs (BCFAs, i.e., *iso*-butyrate and *iso*-valerate) were significantly lower for INF than CON lambs (Table 3). BCFAs are formed

in the rumen by the deamination of amino acids, and their concentrations depend on the ruminal degradation of dietary proteins [47]. The relative proportion of proteins in the abomasum obtained from feed and microbial proteins synthesized in the rumen, however, depended on the physiological status of lambs and diet composition.

Our results (Experiment 1) indicated increased protozoan, bacterial, and methanogenic populations (i.e., *Archaea*, *Methanobacteriales*, and *Methanomicrobiales*) in GIN-infected lambs (Table 4). Infection with the GIN *H. contortus* altered the composition and diversity of the microbial community, which likely facilitated bacterial survival and reproduction. An increase in the abundance of bacterial genera associated with methanogenesis, and other ruminal microorganisms involved in microbial homeostasis, may be affected by infection [48,49]. The ruminal microbiome may therefore be affected by decreasing butyrate and increasing ammonia-N concentrations in GIN-infected lambs, which would affect microorganisms and the genes controlling the metabolic pathway involved in microbial homeostasis. The high ammonia-N concentrations in GIN-infected lambs would consequently inhibit acetoclastic methanogens much more than hydrogenotrophic methanogens, so methane would mainly be formed by hydrogen-using methanogens [50,51]. Acetate oxidation associated with hydrogenotrophic methanogenesis is probably the dominant metabolic pathway for methane formation in GIN-infected animals [52]. However, changes in the ruminal microbiota, including ciliates that are responsible for methanogenesis in GIN-infected animals and 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.

In Experiment 1, GIN infection also affected the specific activities of enzymes of the ruminal microorganisms, especially amylase (Table 4), which is associated with the particulate fraction [53]. Xylanase was higher (not significantly) in GIN-infected lambs because rumen protozoa, fungi, and bacteria are associated with xylanase activities that accelerate the biodegradation of xylan to SCFAs and gases during the processing of lignocellulosic biomass in the rumen [54]. Diet in our study, however, may also have contributed to the increase in amylase activity in the ruminal microorganisms in CON.

In Experiment 2, the dietary substrates containing Herbmix (a mixture of mallow, chamomile, fumitory, and wormwood) [34] or chicory [55] 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 *n*-butyrate and higher ammonia-N and methane concentrations (Table 5). 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 in vitro for estimating ruminal protein degradability [56]. Significantly affected *iso*-butyrate and *iso*-valerate values by substrates without the effect of inocula were probably due to the high protein content of Herbmix and chicory [57,58]. In addition, the ruminal cellulolytic microorganisms could also primarily use BCFAs (i.e., *iso*-butyrate and *iso*-valerate) as the main source of carbon chains for growth.

Diets with a high chicory content ($\geq 70\%$ 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 [14,59,60]. 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 [61]. Our previous results indicated that fermentation in the rumen (in vivo and in vitro) could 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 [62–64]. These bioactive compounds probably

have similar mechanisms of action that also affect GIN larvae [65]. It seems that chicory bioactive compounds may have the potential to inhibit methane production in ruminal fermentation in GIN-infected animals without adversely affecting fermentation. Antimicrobial activity varies amongst flavonoids [66], 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* [67]. Our in vitro results are consistent with either the direct effects of plant bioactive compounds on methanogens or an indirect effect on reducing the production of hydrogen as a substrate for microorganisms [68]. 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 [69]. The effectiveness of bioactive substances, though, can vary considerably depending on the type, source, molecular weight, and dose in the diet [70].

5. Conclusions

We did not confirm the effect of GIN infection on daily weight gain, as feeding lambs with quality feed ensured that lambs were free from adverse effects on growth performance. However, our study confirmed the effect of endoparasites on fermentation and microbial population in the rumens of the lambs. Chicory has promise for mitigating ruminal methane production in vitro and may modulate the ruminal microbial fermentation of GIN-infected lambs. However, the mechanisms and contributions of Herbmix, chicory, and gastrointestinal nematodes to the ruminal microbiome of lambs 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.Š.; investigation, K.M.; resources, K.M.; data curation, A.C. and M.V.; 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: The authors are grateful to Valéria Venglovská and Peter Jerga for their technical support. The English has been revised throughout the manuscript by a native English language editor, William Blackhall.

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.; et al. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. *Prev. Vet. Med.* **2020**, *182*, 105103. [CrossRef] [PubMed]
2. Houdijk, J.G.M.; Tolamp, 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. [CrossRef]

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. [CrossRef] [PubMed]
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.; et al. 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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef] [PubMed]
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. [CrossRef]
9. Zaiss, M.M.; Harris, N.L. Interactions between the intestinal microbiome and helminth parasites. *Parasite Immunol.* **2016**, *38*, 5–11. [CrossRef]
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. [CrossRef]
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. [CrossRef] [PubMed]
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. [CrossRef]
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. [CrossRef] [PubMed]
14. Mravčáková, D.; Komáromyová, M.; Babják, M.; Urda Dolinská, M.; Königová, A.; Petrič, D.; Čobanová, K.; Ślusarczyk, S.; Cieslak, A.; Várady, M.; et al. Anthelmintic activity of wormwood (*Artemisia absinthium* L.) and mallow (*Malva sylvestris* L.) against *Haemonchus contortus* in sheep. *Animals* **2020**, *10*, 219. [CrossRef] [PubMed]
15. Mravčáková, D.; Sobczak-Filipiak, M.; Váradyová, Z.; Kucková, K.; Čobanová, K.; Maršík, P.; Tauchen, J.; Vadlejš, J.; Mickiewicz, M.; Kaba, J.; et al. Effect of *Artemisia absinthium* and *Malva sylvestris* on antioxidant parameters and abomasal histopathology in lambs experimentally infected with *Haemonchus contortus*. *Animals* **2021**, *11*, 462. [CrossRef]
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. [CrossRef]
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. [CrossRef]
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. [CrossRef]
19. Váradyová, Z.; Písaříková, J.; Babják, M.; Hodges, A.; Mravčáková, D.; Kišidayová, S.; Königová, A.; Vadlejš, J.; Várady, M. Ovicidal and larvicidal activity of extracts from medicinal plants against *Haemonchus contortus*. *Exp. Parasitol.* **2018**, *195*, 71–77. [CrossRef]
20. Váradyová, Z.; Mravčáková, D.; Babják, M.; Bryszak, M.; Grešáková, L.; Čobanová, K.; Kišidayová, S.; Plachá, I.; Königová, A.; Cieslak, A.; et al. Effects of herbal nutraceuticals and/or zinc against *Haemonchus contortus* in lambs experimentally infected. *BMC Vet. Res.* **2018**, *14*, 78. [CrossRef]
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. [CrossRef] [PubMed]
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. 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. [CrossRef] [PubMed]
24. Williams, A.G.; Coleman, G.S. *The Rumen Protozoa*, 1st ed.; Springer: New York, NY, USA, 1992; pp. 1–425.
25. 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. [CrossRef] [PubMed]
26. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
27. Bailey, M.J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257–270. [CrossRef]

28. McDougall, E.I. Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochem. J.* **1948**, *43*, 99–109. [\[CrossRef\]](#)
29. 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. [\[CrossRef\]](#)
30. Váradyová, Z.; Kišidayová, S.; Čobanová, K.; Grešáková, L.; 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. [\[CrossRef\]](#)
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. [\[CrossRef\]](#)
33. Váradyová, Z.; Mravčáková, D.; Holodová, M.; Grešáková, L.; 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. [\[CrossRef\]](#)
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. [\[CrossRef\]](#) [\[PubMed\]](#)
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. [\[CrossRef\]](#)
36. Sutherland, I.; Scott, I. *Gastrointestinal Nematodes of Sheep and Cattle: Biology and Control*; Wiley-Blackwell: West Sussex, UK, 2010; pp. 1–242.
37. 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. [\[CrossRef\]](#)
38. 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. [\[CrossRef\]](#)
39. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Torres-Acosta, J.F.J.; Sandoval-Castro, C.A.; Hoste, H.; Aguilar-Caballero, A.J.; Cámara-Sarmiento, R.; Alonso-Díaz, M.A. Nutritional manipulation of sheep and goats for the control of gastrointestinal nematodes under hot humid and subtropical conditions. *Small Rumin. Res.* **2012**, *103*, 28–40. [\[CrossRef\]](#)
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, USA, 2001.
42. Coop, R.L.; Holmes, P.H. Nutrition and parasite interaction. *Internat. J. Parasitol.* **1996**, *26*, 951–962. [\[CrossRef\]](#)
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. [\[CrossRef\]](#)
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. [\[CrossRef\]](#)
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. [\[CrossRef\]](#)
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. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Allison, M.J. Production of branched-chain volatile fatty acids by certain anaerobic bacteria. *Appl. Environ. Microbiol.* **1978**, *35*, 872–877. [\[CrossRef\]](#) [\[PubMed\]](#)
48. 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.; et al. Tannin supplementation modulates the composition and function of ruminal microbiome in lambs infected with gastrointestinal nematodes. *FEMS Microbiol. Ecol.* **2020**, *96*, fiae024. [\[CrossRef\]](#) [\[PubMed\]](#)
49. 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 colubriformis* infection on the ruminal microbiome of lambs. *Exp. Parasitol.* **2021**, *231*, 108175. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Karakashev, D.; Batstone, D.J.; Angelidaki, I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microbiol.* **2005**, *71*, 331–338. [\[CrossRef\]](#)
51. 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*, 5138–5141. [\[CrossRef\]](#)
52. 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*, e0226243. [\[CrossRef\]](#)
53. Raffrenato, E.; Badenhurst, 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. [\[CrossRef\]](#)

54. Takizawa, S.; Asano, R.; Fukuda, Y.; Baba, Y.; Tada, C.; Nakai, Y. Shifts in xylanases and the microbial community associated with xylan biodegradation during treatment with rumen fluid. *Microb. Biotechnol.* **2022**, *15*, 1729–1743. [\[CrossRef\]](#)
55. 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*, 718–726. [\[CrossRef\]](#)
56. 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. [\[CrossRef\]](#)
57. 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. [\[CrossRef\]](#)
58. Petrič, D.; Mravčáková, D.; Kucková, K.; Kišidayová, S.; Cieslak, A.; Szumacher-Strabel, M.; Huang, H.; Kolodziejski, P.; Lukomska, A.; Slusarczyk, S.; et al. Impact of zinc and/or herbal mixture on ruminal fermentation, microbiota, and histopathology in lambs. *Front. Vet. Sci.* **2021**, *8*, 630971. [\[CrossRef\]](#)
59. 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. [\[CrossRef\]](#)
60. 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. [\[CrossRef\]](#)
61. Popp, D.; Plugge, C.M.; Kleinstuber, 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*, e00438–17. [\[CrossRef\]](#)
62. 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. [\[CrossRef\]](#)
63. 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.
64. 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. [\[CrossRef\]](#)
65. 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. [\[CrossRef\]](#)
66. Oskoueian, E.; Abdullah, N.; Oskoueian, A. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Res. Int.* **2013**, *2013*, 349129. [\[CrossRef\]](#)
67. 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. [\[CrossRef\]](#)
68. 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. [\[CrossRef\]](#)
69. 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. [\[CrossRef\]](#)
70. 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. [\[CrossRef\]](#)

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.