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Effects of *Urtica dioica*, *Matricaria chamomilla*, and *Vitex agnus-castus* extracts on *in vitro* rumen fermentation under normal and acidosis conditions

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Abstract: The aim of this study was to investigate the effects of dry extracts of *Urtica dioica*, *Matricaria chamomilla*, and *Vitex agnus-castus* with high phenolic contents on rumen microbial fermentation as compared with those of monensin, a common ionophore antibiotic, using Rumen Simulation Technique (RUSITEC) under normal and acidosis conditions. The treatments were as follows: negative control (no additive), positive control (5 mg/d monensin), and extracts of *U. dioica* (500 mg/d), *M. chamomilla* (500 mg/d), and *V. agnus-castus* (500 mg/d). Neither the plant extracts nor monensin altered the ruminal pH under normal or acidosis conditions. All the treatments affected total volatile fatty acid (VFA) production, propionate production, and dry matter digestibility (DMD), regardless of the fermentation conditions. All three extracts increased (P<0.05) total VFA production similar to that observed with monensin (P<0.05). *M. chamomilla* and *V. agnus-castus* increased propionate production and DMD similar to that obtained with monensin (P<0.05). In contrast to the monensin treatment, all three extracts increased acetate production under normal conditions (P<0.05). Under acidosis conditions, acetate production remained unchanged in the *U. dioica* and *V. agnus-castus* treatments, as well as in the monensin treatment. Under both conditions, the acetate-to-propionate (A:P) ratio decreased only in the monensin treatment (P<0.05). *U. dioica* and *M. chamomilla* had antiprotozoal effects (P<0.05) similar to those of monensin, regardless of the condition. The NH_3-N concentration declined only in the *V. agnus-castus* treatment under acidosis conditions (P<0.05). Similar to the monensin treatment, lactate concentrations remained unchanged in the *V. agnus-castus* treatment under both conditions. In conclusion, plant extracts stimulated fermentative activity of rumen microorganisms under normal and acidosis conditions. Although they did not improve ruminal pH, *U. dioica* and *V. agnus-castus* extracts had more favorable effects on some fermentation parameters under acidosis conditions.

Keywords: Acidosis, plant extracts, rumen fermentation, RUSITEC.

Urtica dioica, *Matricaria chamomilla* ve *Vitex agnus-castus* ekstraktlarının normal koşullar ve asidoz koşulları altında rumen fermentasyonuna *in vitro* etkileri

Özet: Bu çalışmada, *Urtica dioica*, *Matricaria chamomilla* ve *Vitex agnus-castus*'un yüksek fenolik içerikli kuru ekstraktlarının normal koşullar ve asidoz koşulları altında rumen mikrobiyal fermentasyonu üzerine monensin ile karşılaştırmalı etkilerinin Rumen Similasyon Tekniği (RUSITEC) kullanılarak araştırılması amaçlanmıştır. Deneme grupları, negatif kontrol (katkı maddesi yok), pozitif kontrol (5 mg/gün monensin) ve *U. dioica* (500 mg/gün), *M. chamomilla* (500 mg/gün) ve *V. agnus-castus* (500 mg/gün) ekstraktlarından oluşmuştur. Bitki ekstraktları ve monensin ruminal pH'yi normal koşullar ve asidoz koşulları altında değiştirmemiştir. Deneme gruplarının toplam uçucu yağ asidi (UYA) ve propiyonat üretimi ile kuru madde sindirilebilirliği (KMS) üzerine etkilerinin koşuldan bağımsız olarak gerçekleştiği gözlenmiştir. Üç ekstrakt da monensin'e benzer şekilde toplam UYA üretimini arttırmıştır (P<0,05). *M. chamomilla* ve *V. agnus-castus*, propiyonat üretimi ve KMS'yi monensin'e benzer şekilde arttırmıştır (P<0,05). Monensin'in aksine, normal koşullar altında her üç ekstrakt da asetat üretimini arttırmıştır (P<0,05). Asidoz koşulları altında ise asetat üretimi monensin'in yanı sıra *U. dioica* ve *V. agnus-castus* gruplarında da değişmeden kalmıştır. Asetatın propiyonata oranı (A:P), her iki koşulda da sadece monensin grubunda azalmıştır (P<0,05). *U. dioica* ve *M. chamomilla* koşuldan bağımsız olarak monensin'e benzer şekilde antiprotozoal etkiler göstermişlerdir (P<0,05). NH_3-N konsantrasyonu, asidoz koşulları altında sadece *V. agnus-castus* grubunda azalmıştır (P<0,05). Laktat konsantrasyonu, *V. agnus-castus* grubunda her iki koşulda da monensin'e benzer şekilde değişmemiştir. Sonuç olarak, bitki ekstraktları normal koşullar ve asidoz koşulları altında rumen mikroorganizmalarının fermentatif aktivitelerini uyarmıştır. Ruminal pH'yi iyileştirmemiş olmalarına rağmen, *U. dioica* ve *V. agnus-castus* ekstraktları bazı fermentasyon parametreleri üzerine asidoz koşulları altında daha olumlu etkiler oluşturmuşlardır.

Anahtar sözcükler: Asidoz, bitki ekstraktları, rumen fermentasyonu, RUSITEC.

Introduction

Sub-therapeutic doses of ionophore antibiotics have been used since the 1970s to avoid ruminal energy and nitrogen losses and to control metabolic disorders, including acidosis, by selectively inhibiting Gram-positive rumen bacteria and protozoa (29). The use of antibiotics as feed additives was banned in the European Union as of 21 January 2006 due to antibiotic residues in animal products and the development of bacterial resistance (27). Following the ban, there has been intense interest in the development of safer antimicrobial agents that can serve as alternatives to antibiotics as feed additives. Most recent studies have focused on plant extracts and secondary bioactive plant metabolites due to their potential to modify ruminal fermentation (4, 18). However, experimental data on the effects of plant extracts on rumen microbial fermentation under acidosis conditions particularly following normal conditions as in the practice are scarce. Such data would reveal the potential of plant extracts to prevent acidosis.

Urtica dioica (stinging nettle), *Matricaria chamomilla* (chamomile), and *Vitex agnus-castus* (chasteberry) extracts have been used for centuries in traditional medicine and industrial applications, as they contain antimicrobial phenolic compounds, mainly flavonoids (i.e., isorhamnetin, kaempferol, quercetin, rutin, apigenin, and luteolin) and phenolic acids (i.e., caffeic acid, formic acid, malic acid, and chlorogenic acid) (17, 26, 28). In previous studies, extracts of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* were more effective against Gram-positive bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* spp., and *Enterococcus* spp. than Gram-negative bacteria (2, 11, 20), similar to those of ionophore antibiotics, suggesting that these plant extracts may have potential to modify ruminal fermentation. There are some reports on regulatory effects of *U. dioica* on ruminal pH (21) and in the fermentation process of sausage (22) and a few studies on the effects of other dry extracts on various ruminal fermentation parameters under normal rumen conditions (16, 18).

The use of disease models, such as acidosis, that have a negative effect on animal well-being is problematic under *in vivo* conditions due to ethical issues. The standardized semi-continuous Rumen Simulation Technique (RUSITEC) offers an appropriate alternative to such disease models. Therefore, the objective of the present study was to investigate the effects of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts as compared with those of monensin, a commonly used ionophore antibiotic, on *in vitro* rumen microbial fermentation under normal and acidosis conditions.

Material and Methods

Plant extracts: Dry extracts of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* were supplied by Herbal Extracts Plus Co. Ltd. (Croydon, US). The phenolic contents of the plant extracts are summarized in Table 1.

Table 1. Phenolic compounds of plant extracts (μg/g).

Phenolic compounds	Plant extracts		
	U. dioica	***M. chamomilla***	***V. agnus-castus***
Chlorogenic acid	566.6	394	ND
Caffeic acid	36.9	ND	ND
P-Coumaric acid	10.3	47.7	15.6
O-Coumaric acid	ND	5.6	ND
Syringic acid	ND	38.5	ND
Gallic acid	ND	ND	126.9
Caffeic acid	ND	ND	63.3
Rutin	206.9	ND	ND
Quercetin	263.2	542.9	ND
Apigenin	ND	75.9	ND
Luteolin	ND	ND	344.1

ND: not determined.

Incubation technique: RUSITEC was performed as described by Czerkawski and Breckenridge (13). Ten incubation vessels with a nominal volume of 0.75 L were simultaneously used. Inoculum was obtained from a freshly slaughtered 2-y-old healthy Holstein bull (mean body weight: 500 kg) at a commercial slaughter facility. The inoculum transferred in a warm (39°C) insulated flask for use in the *in vitro* system within 30 min. The ruminal fluid was mixed and filtered through three layers of cheesecloth to partition it into liquid and solid (digesta) fractions. Each fermentation vessel was filled with 750 mL of filtered ruminal fluid. Two nylon bags (80 × 120 mm; 150 μm pore size), one containing 80 g of solid digesta and the other containing 16 g of an experimental diet (12.8 g of barley straw cut into 1-cm lengths and 3.2 g of concentrate on a dry matter basis), were placed in the inner perforated containers at the beginning of the experiment. The concentrate was composed of barley, corn, wheat bran, corn gluten meal, sunflower seed meal, dried sugar-beet pulp, molasses, rice bran, vegetable oil, sodium chloride, sodium bicarbonate, calcium carbonate, and a vitamin-mineral premix. According to information obtained from the owner of the Holstein bull, the animal had been fed barley straw ad libitum and 10 kg of a concentrate diet every morning and evening. The same feedstuffs were used in the *in vitro* incubation trial. The chemical composition of the experimental diet is shown in Table 2. After 24 h, the nylon bags containing the solid

digesta from the rumen were replaced with another feed bag containing a fresh experimental diet. Thereafter, only one feed bag was replaced with a new bag daily, and the other bag remained in the system for a further 24 h. Therefore, each feed bag remained in the fermentation vessel for 48 h. The fermentation vessels were maintained at a constant temperature (39°C) and received a continuous infusion of buffers at a rate of 750 mL/d. The chemical composition of the buffer solutions is shown in Table 3. Pure CO_2 was applied to the fermenters when changing the feed bags for continuity of anaerobic conditions.

Table 2. Chemical composition of the experimental diet.

	Barley straw	Concentrate
Dry matter (g/kg)	941.5	927.5
Crude protein (g/kg DM)	37.17	153.10
Crude fat (g/kg DM)	15.93	40.97
Crude fiber (g/kg DM)	445.03	80.86
Ash (g/kg DM)	83.90	78.71
Acid detergent fiber (g/kg DM)	547	-
Metabolizable energy (MJ/kg DM)	6.29	12.10

DM: Dry matter.

Table 3. Chemical composition of the buffer solutions (g/L).

Chemicals	Adaptation period and normal conditions	Acidosis conditions
NaCl	0.470	0.470
KCl	0.570	0.570
$CaCl_2.2H_2O$	0.053	0.053
$MgCl_2.6H_2O$	0.128	0.128
$Na_2HPO_4.12H_2O$	3.720	0.620
$NaHCO_3$	9.800	2.450
pH	8.6	8.6

Experimental procedure: The experiment lasted 21 days (21 d). The first phase of the study (d 1 to d 7) was considered as an adaptation period for the microorganisms to the *in vitro* conditions. In the second phase of the study (d 8 to d 14), 10 RUSITEC fermenters (vessels) were divided into five groups, with two vessels in each group, to investigate the effects of the plant extracts under normal conditions. The five groups were as follows: group 1, no additives (negative control); group 2, 500 mg/d (667 mg/L) of *U. dioica* extract; group 3, 500 mg/d of *M. chamomilla* extract; group 4, 500 mg/d of *V. agnus-castus* extract; and group 5 (positive control), 5 mg/d of monensin (monensin sodium, Fluka). In the third phase of the study (d 15 to d 21), acidosis was established in the RUSITEC fermenters by changing the forage-to-concentrate ratio to 20:80 and reducing the amount of buffering compounds in artificial saliva solution (15). The same amount of each substance was added to the vessels under acidosis conditions.

Sampling and analytical procedures: The phenolic contents (Table 1) of the plant extracts were quantified using a high-performance liquid chromatography (HPLC) (Shimadzu) device equipped with a photodiode array detector. An Agilent Eclipse XDB-C18 (250 × 4.60 mm) 5 µm column at 30ºC and 0.8 mL/min flow speed was used.

The dry matter (920.36), crude protein (984.13), crude fat (920.39), crude fiber (978.10) and ash (942.05) contents of the experimental diet (Table 2) were analyzed according to the procedure of the Association of Official Analytical Chemists (1). The acid detergent fiber was analyzed according to the criteria of Van Soest et al. (32). All samples were ground finely before the chemical analysis.

The pH values were measured daily in each fermentation vessel at the time of feeding using an epoxy body pH electrode (WD-35801-00, Oakton) connected to a pH meter (Ion 6; Acorn series, Oakton). The overflow flasks in the RUSITEC system were placed on ice throughout the experiment to halt microbial activity and to preserve the fermentation products. The liquid effluent was collected daily for VFA, lactate, and NH_3-N determination. Effluents (5 mL) taken for VFA and lactate analysis were stored at -20°C after adding 90 µL of 12 N H_2SO_4. Samples for NH_3-N analysis were frozen directly after collection. The ruminal samples were allowed to thaw completely at 4°C before the analysis. The VFA and lactate concentrations were quantified by HPLC as described previously (14). The NH_3-N concentration was determined with indophenol blue method using the spectrophotometer (UV-150-02; Shimadzu) at 546 nm (9).

The dry matter was determined by drying the feed bags at 65°C for 48 h. The digestibility of the dry matter after 48 h was calculated as the original dry matter sample weight minus the dry matter residue weight divided by the original sample weight (33).

For protozoa counting, rumen fluid samples were removed from the fermenters daily immediately before substrate exchange. The total number of protozoa was counted as described by Demirtas et al. (14).

Statistical analysis: Statistical analysis was performed using the General ANOVA/MANOVA repeated measures factor design, with three fixed effects: two levels of rumen conditions, five levels of treatments, and seven levels of time course. Statistica 5.5 (StatSoft,

Tulsa, OK, USA) was used for the analysis. The effects of time course on microbial fermentation parameters, except for ruminal pH, were not presented in this article. Data of protozoa were transformed by Log10 before variance analysis (25). Significant differences between the means were analyzed using Duncan multiple range test using MstatC software v 1.4 (Michigan State University, 1989). P value of ≤0.05 was considered statistically significant.

Results

Under normal conditions, no significant differences were observed in the ruminal pH for 7 d, and the pH ranged between 6.93 and 6.99. The ruminal pH significantly decreased during the first 3 d when it was switched to acidosis conditions (P<0.05). The pH was 5.65 on d 18 and remained constant thereafter until the end of acidosis. There were no significant differences in the ruminal pH values in the monensin or plant extract groups under the normal and acidosis conditions (Figure 1).

The effects of the plant extracts and monensin treatments on the VFA profile and DMD are shown in Tables 4 and 5, respectively. All the plant extracts increased acetate production under normal conditions (P<0.05), whereas acetate production remained unchanged in the *U. dioica* and *V. agnus-castus* treatments similar to the monensin treatment under acidosis conditions. Similar to monensin, *U. dioica* and *M. chamomilla* had no significant effect on butyrate production under normal conditions. In contrast, under acidosis conditions, the plant extracts increased butyrate production (P<0.05), while monensin decreased butyrate production (P<0.05). All the treatments affected total VFA and propionate production and DMD, regardless of the fermentation conditions. Propionate production and DMD increased in the *M. chamomilla* and *V. agnus-castus* groups (P<0.05), similar to monensin, but remained unchanged in the *U. dioica* group. Total VFA production also increased within all the additive groups (P<0.05). Under both conditions, only monensin reduced the acetate-to-propionate (A:P) ratio (P<0.05), with no significant change observed in any of the plant extract groups. The total protozoa number decreased in the *U. dioica* and *M. chamomilla* groups (P<0.05) similar to that observed in the monensin group, regardless of the fermentation conditions. Under normal conditions, none of the additives had any effect on the NH_3-N concentration, and only *V. agnus-castus* reduced the NH_3-N concentration under acidosis conditions (P<0.05). Lactate concentrations remained unchanged in the *U. dioica* extract treatment under normal rumen conditions and in the *V. agnus-castus* extract treatment under both conditions, similar to monensin treatment.

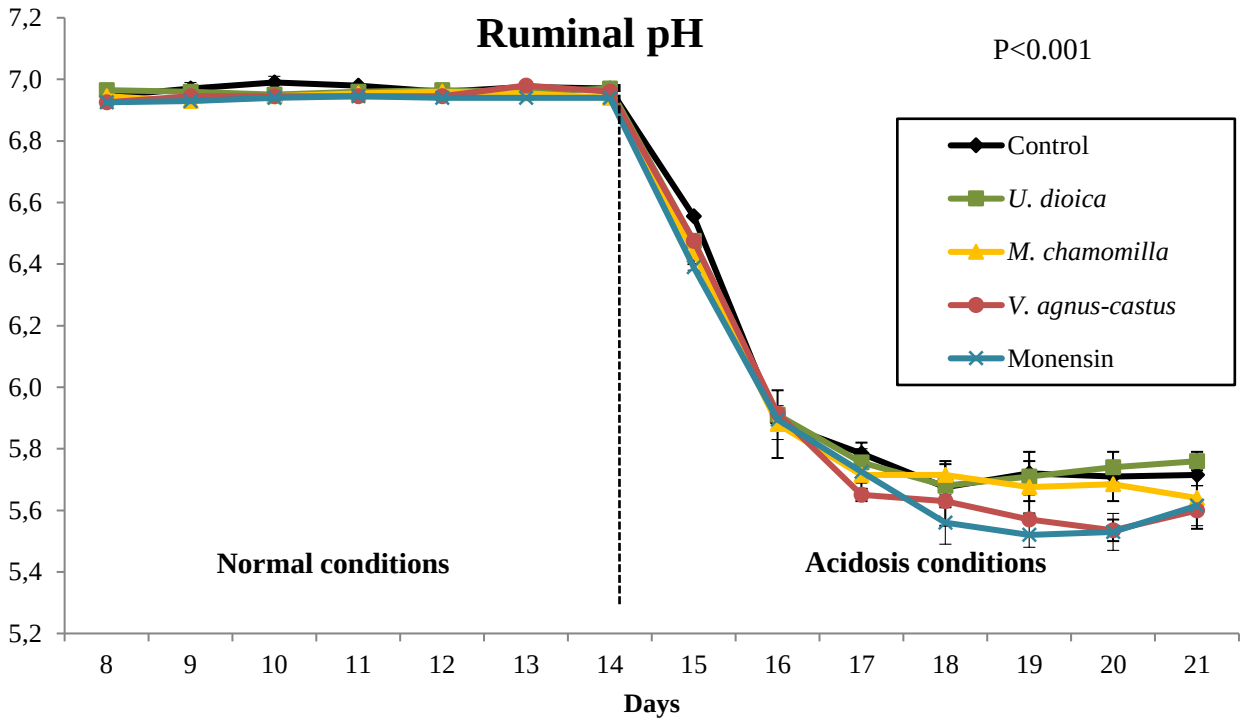

Figure 1. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on ruminal pH during normal and acidosis conditions. Bars indicate standard error. The P value denotes the interaction between rumen conditions and time.

Table 4. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on the production of VFA (mmol/d) and the A:P ratio under normal and acidosis conditions.*

Rumen Condition (RC)	Treatment (T)	Acetate	Propionate	Butyrate	Total VFA	A:P
N	Control (0)	11.89±0.38^{c}	4.33±0.13	2.47±0.09^{e}	18.68±0.58	2.75±0.04^{a}
N	*U. dioica*	13.45±0.36^{b}	5.13±0.16	3.22±0.12^{de}	21.79±0.61	2.63±0.05^{a}
N	*M. chamomilla*	14.47±0.73^{ab}	5.40±0.31	3.44±0.28^{de}	23.31±1.26	2.69±0.04^{a}
N	*V. agnus-castus*	15.47±0.44^{a}	5.63±0.20	3.82±0.11^{d}	24.92±0.73	2.76±0.05^{a}
N	Monensin	11.44±0.58^{cd}	6.54±0.21	2.93±0.17^{de}	20.91±0.71	1.79±0.14^{b}
A	Control (0)	10.33±0.86^{d}	7.77±0.33	7.39±0.48^{b}	25.49±0.75	1.38±0.14^{c}
A	*U. dioica*	10.85±0.98^{cd}	8.00±0.38	8.45±0.50^{a}	27.29±0.84	1.38±0.12^{c}
A	*M. chamomilla*	11.91±0.89^{c}	9.46±0.54	8.54±0.61^{a}	29.91±0.95	1.32±0.12^{c}
A	*V. agnus-castus*	11.41±0.61^{cd}	9.30±0.75	8.80±0.74^{a}	29.51±1.17	1.35±0.14^{c}
A	Monensin	10.16±0.64^{d}	12.63±0.99	5.87±0.44^{c}	28.66±1.42	0.86±0.07^{d}
Main effects						
N		13.34±0.29^{a}	5.40±0.13^{b}	3.18±0.09^{b}	21.92±0.44^{b}	2.53±0.05^{a}
A		10.93±0.36^{b}	9.43±0.35^{a}	7.81±0.28^{a}	28.17±0.50^{a}	1.26±0.06^{b}
	Control (0)	11.11±0.49^{c}	6.05±0.37^{c}	4.93±0.53^{b}	22.09±0.80^{c}	2.06±0.15^{a}
	U. dioica	12.15±0.57^{b}	6.56±0.34^{bc}	5.83±0.56^{a}	24.54±0.73^{b}	2.00±0.14^{a}
	M. chamomilla	13.19±0.62^{a}	7.43±0.50^{b}	5.99±0.59^{a}	26.61±1.00^{ab}	2.01±0.15^{a}
	V. agnus-castus	13.44±0.54^{a}	7.46±0.52^{b}	6.31±0.60^{a}	27.21±0.81^{a}	2.06±0.15^{a}
	Monensin	10.80±0.44^{c}	9.58±0.77^{a}	4.40±0.36^{b}	24.78±1.08^{ab}	1.32±0.12^{b}
P values						
RC		<0.001	<0.001	<0.001	<0.001	<0.001
T		<0.001	0.001	<0.001	0.005	<0.001
RC × T		0.041	0.131	0.021	0.626	0.011

*The values for the main effects of the RC and RC × T are the means of 7 d ± SEM, and the values for the main effects of T are the means of 14 d ± SEM. ^{a,b,c,d,e} Means in the same column followed by different superscripts differ significantly (P<0.05). N: Normal rumen conditions, A: Acidosis conditions, RC: Rumen conditions, T: Treatment, RC × T: Interaction between RC and T, VFA: Volatile fatty acids, A:P: Acetate-to-propionate ratio.

Table 5. Effects of *U. dioica*, *M. chamomilla* and *V. agnus-castus* extracts as compared with those of monensin on DMD coefficients, total protozoa (log 10/mL), and NH_3-N and lactate concentrations (mmol/L) under normal and acidosis conditions.*

Rumen Condition (RC)	Treatment (T)	DMD	Protozoa	NH_3-N	Lactate
N	Control (0)	0.16±0.01	3.22±0.05	0.96±0.07^{c}	0.054±0.015^{cd}
N	*U. dioica*	0.16±0.01	2.89±0.23	1.38±0.18^{c}	0.089±0.016^{c}
N	*M. chamomilla*	0.18±0.01	2.72±0.40	1.41±0.18^{c}	0.131±0.016^{b}
N	*V. agnus-castus*	0.20±0.01	3.37±0.09	1.20±0.12^{c}	0.033±0.014^{d}
N	Monensin	0.18±0.02	2.39±0.35	0.80±0.04^{c}	0.089±0.013^{c}
A	Control (0)	0.37±0.03	1.22±0.39	3.23±0.31^{a}	0.140±0.011^{b}
A	*U. dioica*	0.37±0.02	0.42±0.29	2.93±0.38^{ab}	0.193±0.026^{a}
A	*M. chamomilla*	0.38±0.02	0.00±0.00	2.69±0.28^{ab}	0.186±0.016^{a}
A	*V. agnus-castus*	0.38±0.02	0.20±0.20	2.35±0.20^{b}	0.164±0.016^{ab}
A	Monensin	0.38±0.02	0.00±0.00	3.12±0.22^{a}	0.161±0.019^{ab}
Main effects					
N		0.18±0.01^{b}	2.92±0.12^{a}	1.15 0.06^{b}	0.079±0.008^{b}
A		0.38±0.01^{a}	0.37±0.12^{b}	2.86±0.13^{a}	0.169±0.008^{a}
	Control (0)	0.26±0.03^{c}	2.22±0.27^{a}	2.10±0.27	0.097±0.012^{c}
	U. dioica	0.27±0.02^{bc}	1.65±0.30^{bc}	2.15±0.26	0.141±0.018^{ab}
	M. chamomilla	0.28±0.02^{a}	1.36±0.33^{bc}	2.05±0.21	0.158±0.012^{a}
	V. agnus-castus	0.29±0.02^{a}	1.79±0.32^{ab}	1.78±0.16	0.099±0.017^{c}
	Monensin	0.28±0.02^{ab}	1.19±0.29^{c}	1.96±0.25	0.125±0.013^{b}
P values					
RC		<0.001	<0.001	<0.001	<0.001
T		0.023	0.008	0.445	<0.001
RC × T		0.111	0.183	0.049	0.028

*The values for the main effects of the RC and RC × T are means of 7 d ± SEM, and the values for the main effects of T are means of 14 d ± SEM. ^{a,b,c,d} Means in the same column followed by different superscripts differ significantly (P<0.05). N: Normal rumen conditions, A: Acidosis condition, RC: Rumen conditions, T: Treatment, RC × T: Interaction between RC and T, DMD: Dry matter digestibility.

Discussion and Conclusion

In this study, we investigated the effects of plant extracts on *in vitro* ruminal fermentation parameters under two pH conditions; normal and acidosis. Similar to *in vivo* conditions, acidosis followed normal conditions. One of the aims of the study was to evaluate the potential of the plant extracts to prevent acidosis. Based on our results, neither the plant extracts nor monensin had a significant effect on ruminal pH under normal or acidosis conditions. To the best of our knowledge, there are no reports on the effect of *V. agnus-castus* extract on ruminal pH. Some studies reported that *M. chamomilla* had no effect on ruminal pH (16, 18), as found in the present study. There are a few studies on regulatory effects of *U. dioica* on ruminal pH values under normal rumen conditions. Humphries and Reynolds (21) reported a quadratic increase in *in vivo* ruminal pH values in lactating dairy cows fed a diet supplemented with 10% dried *U. dioica*. However, in their study, *U. dioica* was employed as a whole plant, rather than as an extract; therefore, it was a component of animal ration/substrate by 10%, with a high rate, rather than a feed additive. Active components responsible for antimicrobial action in a sample may vary, depending on how the plant material is used.

In the present study, *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts at a dose of 500 mg/d (about 667 mg/L) stimulated the fermentative activity of rumen microorganisms and resulted in elevated production of total VFA and increased DMD, regardless of the fermentation conditions. The stimulatory effects of *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts on total VFA production and ruminal fermentation at a dose of 500 mg/d (667 mg/L) suggest that these extracts have no toxic effects on ruminal microbes.

The effects of plant extracts used for modifying ruminal fermentation were generally considered positive, when propionate production increased, acetate and butyrate production decreased, and/or the A:P ratio decreased. *M. chamomilla* and *V. agnus-castus* extracts increased propionate production similar to that obtained using monensin, irrespective of the rumen conditions. On the other hand, under normal conditions, all three extracts increased acetate production, whereas monensin did not. Therefore, the A:P ratio decreased only in the monensin treatment. Monensin shows antimicrobial activity against Gram-positive bacteria, which mainly synthesize acetate and butyrate, rather than propionate-producing Gram-negative bacteria (30). In the present study, under normal rumen conditions, all three plant extracts increased acetate production, suggesting that they do not exhibit selective antimicrobial activity against Gram-positive bacteria. Thus, they appear to affect microbial metabolism by a mechanism different from that of monensin.

The *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts used in the present study were rich in flavonoids, such as rutin, quercetin, apigenin, and luteolin, and phenolic acids, such as chlorogenic acid, caffeic acid, coumaric acid, and gallic acid (Table 1). Broudiscou et al. (4) reported that flavonoid-containing dry plant extracts *Lavandula officinalis* and *Solidago virgaure*, administered at a dose of 500 mg/d -as in the present study- increased the production of total VFA and strongly promoted fermentation. Therefore, they have the potential to modify ruminal fermentation. The authors ascribed these effects to the high flavonoid contents of these plant extracts. The effects of flavonoid-rich plant extracts on rumen microorganisms have been attributed to one or a combination of the following hypotheses: *(i)* the inhibitory effects of flavonoids, *(ii)* stimulatory effects of degradation products of flavonoids, and *(iii)* direct actions of other secondary metabolites (5). Interestingly, some studies have also provided support for the second hypothesis which is based on the flavonoids and phenolic acids were hydrolyzed by bacterial enzymes and converted to more bioactive forms which stimulated the enzymatic activity of certain groups of bacteria via the synthesis of aromatic amino acids (3, 24). Cellulolytic bacteria protect themselves against the toxic effects of phenolic compounds in this way and that they use hydrolyzation end-products as a carbon source (10). Greathead (19) classified the stimulatory effect of some herbs and spices on some bacterial species as a prebiotic-type effect and suggested that this effect may be used for manipulating ruminal metabolism (i.e., promoting fiber-digesting bacterial populations). Therefore, the phenolic compounds of plant extracts used in the present study may have generated prebiotic-like effects on some bacterial groups in the rumen, mainly cellulolytic bacteria, considering the increase in the production of acetate under normal rumen conditions.

On the other hand, in the present study, the effects of the treatments on some parameters showed differences in acidosis conditions compared to normal conditions. For example, in the *U. dioica* and *V. agnus-castus* treatment groups, acetate production did not change similar to those of monensin under acidosis conditions but increased under normal conditions. Likewise, the *V. agnus-castus* extract decreased the NH_3-N concentration under acidosis conditions but not under normal rumen conditions. Cardozo et al. (8) reported that the effects of plant extracts on ruminal fermentation might differ, depending on the ruminal pH, and that oregano, garlic, capsicum, yucca extracts, and cinnamaldehyde had more favorable effects on fermentation parameters at pH 5.5 than 7.0. The authors attributed these positive effects to the tendency of active molecules to become undissociated in low pH conditions.

Undissociated forms are more hydrophobic and therefore interact more readily with cell membranes of bacteria and exert antimicrobial effects (8). Active phenolic components of *U. dioica* and *V. agnus-castus* extracts may have inhibitory effects on some strains of Gram-positive bacteria with a similar mechanism, when the ruminal pH is low and have more favorable effects under acidosis than normal rumen conditions.

In the present study, *U. dioica* and *M. chamomilla* extracts exhibited antiprotozoal effects similar to those observed in the monensin treatment, irrespective of the rumen conditions. *U. dioica* and *M. chamomilla* extracts contain rutin, quercetin, and apigenin, in addition to chlorogenic acid, all of which have been reported to have antiprotozoal, antiplasmodial, and antitrypanosomal effects (6, 7, 23, 31). Therefore, flavonoids can interact with microorganisms in a negative, as well as in a positive way (4).

Lactate concentrations remained unchanged in the *U. dioica* extract treatment under normal rumen conditions in the present study and in the *V. agnus-castus* extract treatment under both conditions, similar to monensin treatment. Lactate is an intermediate in rumen metabolism and can be converted to other VFAs or long-chain fatty acids. Previous research reported that 60–95% of lactate produced after concentrate-rich feeding was converted to propionate by the acrylate pathway and that 20–30% was converted to butyrate by *Megasphaera elsdenii* (12). In the present study, lactate might be converted to propionate and butyrate in the *V. agnus-castus* extract group, considering that this extract was unique additive, which increased the production of propionate and butyrate but did not change lactate concentrations under both normal and acidosis conditions.

In conclusion, *U. dioica*, *M. chamomilla*, and *V. agnus-castus* extracts positively affected *in vitro* ruminal fermentation by stimulating the fermentative activity of rumen microorganisms under both normal and acidosis conditions. However, the mode of action of these plant extracts appears to differ from that of monensin, particularly under normal rumen conditions. Although none of the plant extracts prevented acidosis, *U. dioica* and *V. agnus-castus* extracts had more favorable effects on some fermentation parameters such as the NH_3-N concentration and acetate production under acidosis conditions. The effects of higher concentrations of *V. agnus-castus* on lactate production should be studied, although it did not exert prominent effects in the present study. Further *in vivo* studies are required to determine the value of these extracts as feed additives in enhancing the efficiency of ruminal fermentation and animal performance.

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Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Association of Official Analytical Chemists** (AOAC) (2000): Official Methods of Analysis of AOAC International. AOAC, USA.
2. **Arokiyaraj S, Perinbam K, Agastian P, et al** (2009): *Phytochemical analysis and antibacterial activity of Vitex agnus-castus.* Int J Green Pharm, **3**, 162-164.
3. **Aura AM** (2008): *Microbial metabolism of dietary phenolic compounds in the colon.* Phytochem Rev, **7**, 407-429.
4. **Broudiscou LP, Papon Y, Broudiscou AF** (2000): *Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes.* Anim Feed Sci Tech, **87**, 263-277.
5. **Broudiscou LP, Papon Y, Broudiscou AF** (2002): *Effects of dry plant extracts on feed degradation and the production of rumen microbial biomass in a dual outflow fermenter.* Anim Feed Sci Tech, **101**, 183-189.
6. **Calzada F, Alanís AD, Meckes M, et al** (1998): *In vitro susceptibility of Entamoeba histolytica and Giardia lamblia to some medicinal plants used by the people of Southern Mexico.* Phytother Res, **12**, 70-72.
7. **Calzada F, Velázquez C, Cedillo-Rivera R, et al** (2003): *Antiprotozoal activity of the constituents of Teloxys graveolens.* Phytother Res, **17**, 731-732.
8. **Cardozo PW, Calsamiglia S, Ferret A, et al** (2005): *Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle.* J Anim Sci, **83**, 2572-2579.
9. **Chaney AL, Marbach EP** (1962): *Modified reagents for determination of urea and ammonia.* Clin Chem, **8**, 130-132.
10. **Chesson A, Colin SS, Wallace RJ** (1982): *Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria.* Appl Environ Microb, **44**, 597-603.
11. **Cinco M, Barfi E, Tubaro A, et al** (1983): *A microbial survey on the activity of a hydroalcholic extract of Cammomile.* Int J Crude Drug Res, **21,** 145-151.
12. **Counotte GH, Prins RA, Janssen RAM, et al** (1981): *Role of Megasphaera elsdenii in the fermentation of DL-[2-^{13}C] lactate in the rumen of dairy cattle.* Appl Environ Microb, **42**, 649-655.
13. **Czerkawski JW, Breckenridge G** (1977): *Design and development of a long term rumen simulation technique (Rusitec).* Brit J Nutr, **38**, 371-384.
14. **Demirtas A, Ozturk H, Sudagidan M, et al** (2019): *Effects of commercial aldehydes from green leaf volatiles (green odour) on rumen microbial population and fermentation*

profile in an artificial rumen (Rusitec). Anaerobe, **55**, 83-92.

15. **Gakhar N** (2008): *Development of alternate markers of subacute ruminal acidosis (SARA).* Master's thesis, University of Manitoba, Manitoba, Canada.
16. **Garćia-Gonźalez R, López S, Fernández M, et al** (2008): *Screening the activity of plants and spices for decreasing ruminal methane production in vitro.* Anim Feed Sci Tech, **147**, 36-52.
17. **Gardiner P** (2000): Chasteberry (*Vitex agnus castus*). http://online.fliphtml5.com/ojsg/pwqv/#p=1. (8 May 2019).
18. **Ghasemifard M, Rahchamani R, Ghanbari F, et al** (2017): *Effects of Matricaria chamomille and Cichorium intybus powder on performance, rumen microbial population and some blood parameters of Dallagh sheep.* Iran J Vet Med, **11**, 267-277.
19. **Greathead H** (2003): *Plants and plant extracts for improving animal productivity.* P Nutr Soc, **62**, 279-290.
20. **Gülçin I, Küfrevioğlu OI, Oktay M, et al** (2004): *Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (Urtica dioica L.).* J Ethnopharmacol, **90**, 205-215.
21. **Humphries DJ, Reynolds CK** (2014): *The effect of adding stinging nettle (Urtica dioica) haylage to a total mixed ration on performance and rumen function of lactating dairy cows.* Anim Feed Sci Tech, **189**, 72-81.
22. **Kaban G, Aksu Mİ, Kaya M** (2008): *Effect of Urtica dioica L. on the growth of Staphylococcus aureus in traditional dry fermented sausage.* J Muscle Foods, **19**, 399-409.
23. **Köhler I, Jenett-Siems K, Siems K, et al** (2002): *In vitro antiplasmodial investigation of medicinal plants from El Salvador.* Z Naturforsch, **57**, 277-281.
24. **Lampe JW, Chang JL** (2007): *Interindividual differences in phyto-chemical metabolism and disposition.* Semin Cancer Biol, **17**, 347-353.
25. **McDonald JH** (2008): Data transformations. 148-152. In: JH McDonald (Ed), Handbook of Biological Statistics. Sparky House Publishing, Baltimore.
26. **Mckay DL, Blumberg JB** (2006): *A review of the bioactivity and potential health benefits of chamomile tea (Matricaria recutita L.).* Phytother Res, **20**, 519-530.
27. **OJEU** (2003). OJEU *Regulation (EC) No 1831/2003 of the European Parliament and the Council of 22 September 2003 on Additives for Use in Animal Nutrition.* Official Journal of European Union. Page L268/36 in OJEU of 18/10/2003.
28. **Pinelli P, Ieri F, Vignolini P, et al** (2008): *Extraction and HPLC analysis of phenolic compounds in leaves, stalks, and textile fibers of Urtica dioica L.* J Agr Food Chem, **56**, 9127-9132.
29. **Russell JB, Houlihan AJ** (2003): *Ionophore resistance of ruminal bacteria and its potential impact on human health.* FEMS Microbiol Rev, **27**, 65-74.
30. **Stewart CS** (1991): The Rumen Bacteria. 15-26. In: JP Jouany (Ed), The Rumen Microbial Metabolism and Ruminant Digestion. INRA Editions, Paris.
31. **Tasdemir D, Brunb R, Franzblau SG, et al** (2008*): Evaluation of antiprotozoal and antimycobacterial activities of the resin glycosides and the other metabolites of Scrophularia cryptophila.* Phytomedicine, **15**, 209-215.
32. **Van Soest PJ, Robertson JB, Lewis BA** (1991): *Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition.* J Dairy Sci, **74**, 3583-3597.
33. **Zhang HL, Chen Y, Xu XL, et al** (2013): *Effects of branched-chain amino acids on in vitro ruminal fermentation of wheat straw.* Asian-Australas J Anim Sci, **26,** 523-528.