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Research Article

Effects of Cleavers *(Galium aparine)* and Yarrow *(Achillea millefolium)* Extracts on Rumen Microbial Fermentation in *In-vitro* Semi-Continuous Culture System (RUSITEC)

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Abstract

Experimental data on the effects of cleavers *(Galium aparine)* and yarrow *(Achillea millefolium)* extracts on rumen microbial fermentation are scarce. The objective of this study was to determine the effects of cleavers and yarrow extracts on *in vitro* ruminal fermentation. Incubation trial was carried out using the long-term rumen simulation technique (RUSITEC). The experiment lasted 10 days. After an adaptation period of 5 days, the fermentation vessels divided into 3 groups; first three vessels received no additives (control), second three vessels received 500 mg/L cleavers extract daily, and third three vessels received 500 mg/L yarrow extract daily. Supplementations of cleavers and yarrow extracts had no significant effect on ruminal pH, total volatile fatty acids (VFA), acetate, propionate and methane production, NH_3-N concentration and, total protozoa. However, both extracts decreased dry matter digestibility (DMD) ($P<0.05$). Butyrate production, on the other hand, increased with cleavers extract ($P<0.05$). In conclusion, there were only small effects of cleavers and yarrow extracts on the investigated microbial fermentation characteristics. Nevertheless, it may be considered advantageous for feed conversion that plant extracts did not suppress ruminal fermentation in spite of decreasing DMD.

Keywords: *Achillea millefolium, Cleavers, Galium aparine, Plant extracts, Rumen, RUSITEC, Yarrow*

Yoğurt Otu *(Galium aparine)* ve Civan Perçemi *(Achillea millefolium)* Ekstraktlarının *In-vitro* Yarı-Sürekli Kültür Sisteminde (RUSITEC) Rumen Mikrobiyal Fermentasyonu Üzerine Etkileri

Öz

Yoğurt otu *(Galium aparine)* ve civan perçemi *(Achillea millefolium)* ekstraktlarının rumen mikrobiyal fermentasyonu üzerine etkileri ile ilgili sınırlı düzeyde deneysel veri bulunmaktadır. Bu çalışmanın amacı, yoğurt otu ve civan perçemi ekstraktlarının *in vitro* ruminal fermentasyon üzerine etkilerinin araştırılmasıdır. Inkübasyon denemesi uzun-süreli rumen simülasyon tekniği (RUSITEC) kullanılarak gerçekleştirilmiştir. Deneme 10 gün sürmüştür. Beş günlük bir adaptasyon periyodunu takiben fermenterler 3 gruba bölünmüştür; ilk üç fermentere ilave yapılmamış (kontrol), ikinci üç fermenterlik gruba günlük 500 mg/L yoğurt otu ekstraktı, üçüncü üç fermenterlik gruba ise günlük 500 mg/L civan perçemi ekstraktı eklenmiştir. Yoğurt otu ve civan perçemi ilaveleri, ruminal pH, toplam uçucu yağ asitleri (UYA), asetat, propiyonat ve metan üretimi, NH_3-N konsantrasyonu ve toplam protozoa üzerine önemli bir etki oluşturmamıştır. Ancak her iki ekstrakt da kuru madde sindirilebilirliğini (KMS) azaltmıştır ($P<0.05$). Diğer taraftan, bütirat üretimi, yoğurt otu ekstraktı ilavesi ile artış göstermiştir ($P<0.05$). Sonuç olarak, incelenen mikrobiyal fermentasyon özellikleri üzerine yoğurt otu ve civan perçemi ekstraktları sadece küçük etkiler oluşturmuşlardır. Bununla birlikte, bitki ekstraktlarının KMS'yi azaltmalarına rağmen ruminal fermentasyonu baskılamamış olmalarının, yem maddelerinin değerlendirilmesi açısından avantaj olarak kabul edilebileceği düşünülmektedir.

Anahtar sözcükler: *Achillea millefolium, Bitki ekstraktları, Civan perçemi, Galium aparine, Rumen, RUSITEC, Yoğurt otu*

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INTRODUCTION

Manipulation of rumen microbial fermentation for enhancing feed digestibility, mitigating methane emission and nitrogen excretion by ruminants to improve animal productivity and to lower product cost is one of the main aims of nutritional strategies. Stabilizing ruminal pH to reduce the incidence of metabolic disorders such as subacute ruminal acidosis has long been another target for the rumen nutritionists [1]. Antibiotic feed additives which selectively inhibit Gram-positive rumen bacteria and rumen protozoa have been successfully used in ruminant rations for these purposes [2]. However, the risk of residues in animal products as well as the concern about the appearance of resistant strains of bacteria led to the prohibition of antibiotic use in animal feeds in the European Union [3] and Turkey [4] since 2006. Accordingly, the focus of researchers has shifted to the study of natural alternatives such as plant extracts and their secondary metabolites to manipulate ruminal fermentation in order to improve ruminant productivity [5]. Many of the plant secondary compounds in the extracts are well defined as antimicrobial agents which act against bacteria, protozoa and fungi [6]. In a new study, we have observed that green leaf volatiles which are derived from unsaturated fatty acids by plants with green leaves had favorable effects on protein utilization in the rumen via suppressing rumen protozoa [7].

Cleavers *(Galium aparine)* and yarrow *(Achillea millefolium)* are important medicinal plants which have been used for centuries to treat various diseases [8,9]. Cleavers grows widespread in Anatolia [8] and extracts of this plant are widely used in the treatment of sepsis, skin infections, and infections of respiratory and genitourinary systems due to antimicrobial properties [10]. The main biologically active antimicrobial phenolic substances in cleaver extract were chlorogenic acid, caffeic acid, and rutin [11]. It has been reported that Gram-positive bacteria mainly *Staphylococcus aureus* and *Bacillus subtilis* are more sensitive to cleavers extract than Gram-negative species such as *Escherichia coli* and *Proteus vulgaris* [10]. Yarrow also has antimicrobial activity and thus it is one of the most commonly used herbs in Europe in traditional animal healthcare and livestock production. Gastroprotective, antiulcerogenic, and anti-inflammatory properties of yarrow extract have been reported [9]. Yarrow extract was rich in antimicrobial phenolic compounds such as chlorogenic acid, vicenin-2, luteolin-7-*O*-glucoside, rutin, apigenin-7-*O*-glucoside, luteolin, and apigenin [12]. *Staphylococcus aureus* and *Streptococcus pneumoniae* among Gram-positive wound pathogens have been reported to be more sensitive to yarrow extract than *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* as Gram-negative pathogen species [13]. Yarrow essential oil has been also reported to be an effective antimicrobial agent against *Listeria* species [14] and a protozoan parasite *Trypanosoma cruzi* [15]. Antibiotic feed additives such as monensin produce desirable effects on rumen fermentation by showing selective antimicrobial activity on Gram-positive bacteria and protozoa in the rumen [2]. Therefore, the above-reported effects of these plant extracts on Gram-positive bacteria and protozoa suggest that they may be alternatives to antibiotics as feed additives in the modification of ruminal fermentation if they act in the same way in the rumen. However, there is limited information about the effects of yarrow extract on rumen fermentation. The ability of cleavers extract to influence microbial fermentation processes in the rumen have not been evaluated. Therefore, the aim of this study was to investigate the effects of cleavers and yarrow extracts on rumen microbial fermentation in an *in vitro* semi-continuous culture system (RUSITEC).

MATERIAL and METHODS

Plant Extracts

The extracts were provided by Kale Naturel Herbal Products Company, Ltd., Balıkesir, Turkey. As specified by the manufacturer, plant samples were air dried, ground in a mill into 0 to 200 µm large particles and screened. Powdered plant materials were extracted with 80% ethanol (1/10, w/v) at 30°C for 4-5 h and filtered to give homogenous liquid. The extracts was reduced to 1/5 of its volume using a rotary vacuum evaporator at 35°C for 8 h and dried in a laboratory scale spray-dryer. Afterwards, dry extracts liquefied in the mixer at an adequate ratio.

Analyses of Phenolic Compounds of Plant Extracts

Phenolic compounds *(Table 1)* of cleavers and yarrow 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 30°C and 0.8 mL/min flow speed was used.

Artificial Rumen System

The present study was carried out using the rumen simulation technique (RUSITEC) as described by Czerkawski and Breckenridge [16] applying slight modification according to Oeztuerk et al.[17]. The complete unit consisted of nine 1 L volume anaerobic fermenters. The inoculum was obtained from two freshly slaughtered beef cattle (400 kg mean body weight) at a commercial slaughter facility and transferred in warm (39°C) insulated flasks to the *in vitro* system within 30 min. According to information obtained from the owner, the animals had been fed a diet (8.0 kg DM/d) consisting of 20% barley straw and 80% commercial mineral- and vitamin supplemented concentrate for growing cattle. The same diet was also used for *in vitro* incubation trials *(Table 2)*. The commercial concentrate consisted of corn, wheat bran, corn gluten feed, molasses, sunflower seed meal, barley, distilled corn grain residues, soya bean meal,

Table 1. *Phenolic compounds of cleavers (G. aparine) and yarrow (A. millefolium) extracts*

Phenolic Compounds (ppm)	Cleavers Extract	Yarrow Extract
Gallic acid	ND	4.7
Protocatechuic acid	1.7	70.0
P-hydroxy benzoic acid	23.6	13.8
Chlorogenic acid	10.5	72.2
Caffeic acid	13.3	65.9
Syringic acid	2.4	13.0
Vanilin	1.6	18.6
P-coumaric acid	1.9	9.0
Ferulic acid	1.1	27.6
Benzoic acid	30.1	29.3
O-coumaric acid	0.6	6.7
Eriodictiol	ND	36.2
Apigenin	23.6	43.8
ND: not determined		

Table 2. *Chemical composition of experimental diet*

Nutrients, %	Barley Straw	Concentrate
Dry matter	94.19	91.12
Ash	5.82	6.50
Crude fiber	40.45	5.56
Crude protein	2.95	12.42
Ether extract	2.00	2.75
Organic matter	88.37	84.62
None fiber carbohydrates	16.43	61.44
Neutral detergent fiber	72.80	16.89
Acid detergent fiber	49.98	7.41
Metabolizable energy (MJ/kg)	6.28	11.11

vinasse, vegetable oil, calcium carbonate, sodium chloride, and a vitamin-mineral premix. Ruminal fluid was filtered through four layers of cheesecloth to partition into liquid and solid (digesta) fractions. Each fermentation vessel was filled with 750 mL of filtered ruminal fluid. Squeezed solid digesta (80 g) was weighed into a nylon bag (80 × 120 mm; 150 µm pore size), and placed in the inner perforated containers together with 10 g of an experimental diet (5 g barley straw and 5 g concentrate on feed basis). After 24 h, the solid digesta bag was withdrawn and a bag with feed was supplied. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermentation vessel for 48 h. Fermentation vessels received a continuous infusion of a buffer (pH 7.4) at a rate of 750 mL/d [17]. The chemical composition of the buffer solution is presented in *Table 3*.

Experimental Design

The experiment was conducted as a completely randomized design (CRD) with three treatments and three replicates per treatment. The incubation trial consisted of a 5-day adaptation period (to achieve steady-state conditions) followed by a 5-day collection period. At the start of the collection period, the commercial extracts of cleavers *(G. aparine)* or yarrow *(A. millefolium)* were added to the respective fermentation vessels. Although there is no literature that evaluates effects of cleavers extract on rumen fermentation, yarrow extract was supplied at the rate of 500 mg per day in 1 L dual outflow fermenters by Broudiscou et al.[18]. Therefore, 500 mg/L dose of both extracts were used in the present study. During the collection period, the 9 vessels were divided into 3 groups: three of them received daily 500 mg/L of cleavers extract, three vessels received daily 500 mg/L of yarrow extract and three vessels received no additives (control).

Table 3. *Chemical composition of the buffer solution*

Chemicals	mmol/L
NaCl	28.00
KCl	7.69
1N HCl	0.50
$CaCl_2 \cdot 2H_2O$	0.22
$MgCl_2 \cdot 6H_2O$	0.63
$Na_2HPO_4 \cdot 12H_2O$	10.00
$NaH_2PO_4 \cdot H_2O$	10.00
$NaHCO_3$	97.90

Chemical Composition Analysis of Feed

The dry matter, crude protein, crude fat, crude cellulose, and ash contents of experimental diets *(Table 2)* were analyzed according to the procedure of the Association of Official Analytical Chemists [19]. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured [20] using an ANKOM^{200} Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA). NDF, using sodium sulfite, heat stable amylase, and ADF were expressed including residual ash. Non-fiber carbohydrates (NFC) were calculated as follows: NFC% = (100% - CP% - NDF% - EE% - Ash%) [21]. Organic matter (OM) content was determined based on the value of dry matter content minus ash content and metabolizable energy according to TSE [22] methods.

Sample Collection and Analytical Procedures

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).

Liquid effluent was collected daily and samples were taken and frozen at -20°C for later analyses for volatile fatty acids (VFA) and NH_3-N. VFA were quantified by the method of Ozturk et al.[23] using HPLC (Shimadzu LC-20AT) with a Rezex ROA-organic acid column (7.8 × 300 mm) at 60°C,

isocratic elution with 0.005 M H_2SO_4, and UV detection at 210 nm. NH_3-N concentration was determined using a colorimetric technique as described by Bhandari et al.[24].

Methane production was calculated using the equations proposed by Abdl-Rahman [25] based on the stoichiometry of Wolin [26] as following:

Fermentative CO_2 = A/2 + P/4 + 1.5 B

Fermentative CH_4 = (A + 2 B) – CO_2

A = mole of acetate

P = mole of propionate

B = mole of butyrate

Dry matter was determined by drying bags at 65°C for 48 h. The digestibility of dry matter at 48 h was calculated as original dry matter sample weight minus dry matter residue weight divided by the original sample weight. This value was then multiplied by 100 to derive the digestibility of dry matter percentage.

For protozoa counting, 1 mL of rumen fluid sample was mixed with 1 mL of a solution of 0.6 g methyl green, 8 g NaCl and 100 mL formaldehyde (37%) filled up to 1000 mL with distilled water. The portions of the samples were, then, pipetted into a counting chamber (Fuchs-Rosenthal: 0.0625 mm^2; 0.2 mm deep; Marienfeld, Germany). Total numbers of protozoa, without quantifying different types, were determined using a light microscope (Leica CME).

Statistical Analyses

Fermentation data were analyzed using two-way repeated measures analysis of variance (two-way ANOVA) with the SigmaStat 3.1 software (Systat Software, Erkrath, Germany). The model included the fixed effects of treatment, time, and their interaction and random effects of fermenters. The individual fermenter was used as the experimental unit for statistical analysis. In case of a significant ANOVA result, post hoc Duncan tests were performed to evaluate the statistical differences between the groups. P values of <0.05 were considered significant.

RESULTS

Effects of cleavers and yarrow extract on *in vitro* ruminal fermentation are given in *Table 4*. Neither cleavers nor yarrow extracts had a significant effect on ruminal pH, total VFA, acetate, propionate and methane production, NH_3-N concentration and, total protozoa. However, both extracts decreased dry matter digestibility (P<0.05). Butyrate production, on the other hand, increased with cleavers extract (P<0.05). Treatment × Time interaction was significant for total protozoa (P=0.004) and NH_3-N concentration (P=0.021). Total protozoa were higher (P<0.05) in the presence of yarrow and cleavers extracts than control on the 1st day of collection period and, on the 1st and 2nd days of the collection period, respectively. Cleavers and yarrow extracts also increased (P<0.05) NH_3-N concentration on the 4th day of the collection period.

DISCUSSION

In the present study, cleavers and yarrow extracts were evaluated for their potential application as modifiers of rumen microbial fermentation. Until recently, no studies have been conducted on the effects of cleavers on rumen microbial fermentation and, there is only limited data about the effects of yarrow on rumen fermentation. The extracts tested in this study showed no significant influence on the culture pH in RUSITEC fermenters. The pH values were within the normal range (6.5-7.0). Published reports on the effect of plant extracts on ruminal pH are variable. The findings of the present study for ruminal pH were agreed with previous researches that found no effect on ruminal pH [27-29]. The diet supplemented with a medicinal plant mixture containing yarrow as a component also did not change the pH of the sheep rumen [30,31].

Table 4. *Effects of cleavers (G. aparine) and yarrow (A. millefolium) extracts (500 mg/L) on ruminal fermentation in RUSITEC*

Parameters	Treatments			SEM	P Value
	Control	Cleavers Extract	Yarrow Extract		T × T
Ruminal pH	6.72	6.74	6.73	0.00	0.818
Total VFA (mmol/d)	27.54	27.93	27.87	1.10	0.861
Acetate	15.75	15.28	15.53	0.64	0.910
Propionate	8.29	8.50	8.56	0.43	0.869
Butyrate	3.51^{b}	4.15^{a}	3.78^{ab}	0.12	0.471
Methane (mmol/d)	7.55	7.60	7.51	0.28	0.870
DMD (%)	56.20^{a}	51.87^{b}	52.05^{b}	0.92	0.156
Protozoa (×10^3/mL)	5.67	10.71	8.63	1.64	0.004
NH_3-N (mmol/L)	6.02	6.36	6.03	0.18	0.021

^{a,b} Means in the same row followed by different superscripts differ significantly (P<0.05). VFA: Volatile fatty acids; DMD: Dry matter digestibility; T × T: Treatment × Time interaction.

Effects of plant extracts on ruminal fermentation are desirable if they increase or do not change total VFA production while the reduced VFA production is generally considered as a sign of depressed microbial fermentation [32]. The production of total VFA was not affected by both plant extracts in the present study. Our result is consistent with the report by Broudiscou et al.[33] in which *A. millefolium* (yarrow) extract at 500 mg/day did not affect the concentration of total VFA in 1 L dual outflow fermenters. Yarrow essential oil also did not change *in vitro* total VFA concentration in the dose range of 0-750 mg/L [34]. Yarrow extract did not change VFA profile in our study although it was reported to increase the molar proportion of acetate to the detriment of butyrate [33]. The conflicting results about VFA profile may be due to differences in the chemical composition of the diets and extracts used in different studies. On the other hand, the increase in butyrate production with the supplementation of cleavers extract in the present study suggests that the cleavers extract favored butyrate-producing bacteria at the used dose. Butyrate-producing bacteria in the rumen are generally in Gram-positive nature [35]. Cleavers extract used in the present study contained several phenolic compounds such as *p*-hydroxy benzoic acid, caffeic acid, benzoic acid, vanillin, and apigenin *(Table 1)*. There are reports about stimulatory effects of phenolic acids and flavonoids on growth and fermentation activity of particularly Gram-positive rumen bacteria [36-38].

The main significant findings of this study were the effects of cleavers and yarrow extracts on dry matter digestibility. Both extracts decreased digestibility of feed dry matter after 48 h incubation in RUSITEC. Broudiscou et al.[18] reported that *A. millefolium* (yarrow) extract depressed moderately *in vitro* organic matter digestibility as well as increasing degradability of crude protein and cell-wall constituents. *In vitro* degradability of dry matter and organic matter decreased by 500, 750 and, 1000 mg/L of yarrow essential oil according to the report by Kahvand and Malecky [34] which was consistent with our findings. However, they did not observe any adverse effect on digestibility at 250 mg/L dose. In the present study, yarrow extract at the used dose might also have inhibitory effects on some cellulolytic bacteria through the phenolic compounds it contained. Nevertheless, surprisingly no inhibition was observed for daily production of VFA. The decrease in dry matter digestibility without affecting the total VFA production may be related to a higher sensitivity and/or resistance of some specific type of cellulolytic bacteria [39]. According to another report, *in vitro* dry matter digestibility was lowest for yarrow after 24 h ruminal incubation [40]. The authors attributed this effect to the lower nitrogen content in comparison with the other examined herbs. Moreover, Hammond et al.[41] also reported that dairy heifers fed with a ryegrass and wild flower mixture of predominately sorrel, ox-eye daisy, yarrow, knapweed, and ribwort plantain had 18% lower DMD compared to control heifers fed with only ryegrass.

Methane production and NH_3-N concentration were also not affected by plant extracts. Compatible with the results of the present study, Broudiscou et al.[33] reported that *A. millefolium* (yarrow) extract at 500 mg/L dose did not have a significant effect on *in vitro* methanogenesis. Yarrow essential oil also did not mitigate *in vitro* ruminal methane production at 0-750 mg/L doses [34]. However, it lowered methane production at 1000 mg/L dose which was accompanied with a concomitant decrease in production of total gas and VFA, microbial biomass, and digestibility of dry matter as well as organic matter indicating a general inhibition of rumen fermentation [34]. The effects of plant extracts on rumen NH_3-N concentration are also dose-dependent and these compounds are more effective when used at high doses compared with at low doses. For example, Castillejos et al.[42] evaluated the effects of increasing doses of vanillin and eugenol on rumen fermentation in a 24 h *in vitro* fermentation and showed that these essential oils decreased NH_3-N concentration at the highest dose (5000 mg/L), but no effects were observed at lower doses.

The numbers of large protozoa were increased by *A. millefolium* (yarrow) extract in dual outflow fermenters according to Broudiscou et al.[33]. However, our extracts had no effect on rumen fluid protozoal counts except the increase in the first few days of the experiment. Type and amount of active components in the extracts might play a role in the expression of stimulatory or inhibitory effects against protozoa.

The present study showed that there were only small effects of cleavers and yarrow extracts on the investigated microbial fermentation characteristics in the semi-continuous rumen simulation technique (RUSITEC). Nevertheless, it may be considered advantageous for feed conversion that plant extracts did not suppress ruminal fermentation in spite of decreasing dry matter digestibility. However, further *in vitro* studies should be conducted using higher doses of these extracts, and *in vivo* studies are needed to evaluate their efficacy as alternative feed additives in ruminant nutrition.

Conflict of Interests

The authors declare no competing interests.

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