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EFFECTS OF A DIET CONTAINING WHOLE COTTONSEED ON RUMEN PROTOZOAL POPULATION AND FERMENTATION PARAMETERS IN SHEEP

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ABSTRACT

In this experiment *in vitro* and *in vivo* trials were performed to investigate the efficacy of whole cottonseed to limit protozoal population and fermentation parameters. Treatments were 1) control (without whole cottonseed) and 2) 20% whole cottonseed (WCS). Dry matter disappearance (DMD) and fermentation characteristics of the treatments were determined in the *in vitro* incubation studies. In the *in vivo* trial, all sheep were fed a control diet for 3 wk, prior to the start of the 28-day experimental period, in a completely randomize design. Ruminal fluid was taken by rumenocentesis (3 h after feeding) on days 1, 3, 5, 7, 9, 11, 14, 21 and 28 from four sheep fed treatment diets. The pH and protozoal counts were determined on each ruminal fluid sample was taken from sheep, while ammonia nitrogen and volatile fatty acids (VFA) were determined in samples taken on days 7, 14, 21 and 28. The *in vitro* DMD at 72 h incubation was higher ($P<0.01$) for the control diet. After 72 h of incubation, the total VFA concentration (mM) ($P<0.05$) and the acetate to propionate ratio ($P<0.05$) was higher for the control diet than WCS diet. After 24 h of incubation, the molar proportion (%) of acetate, propionate and valerate ($P<0.05$) were higher, but the molar proportion of isovalerate was lower ($P<0.05$) for the control diet than for the WCS diet. In *in vivo* study, feeding cottonseed decreased ($P<0.05$) total protozoa population from approximately 450,000 to 240,000 ml^{-1}. Holotrich and cellulolytic protozoa disappeared from the rumen samples of sheep whereas

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Entodinium sp., remained. This was associated with lower concentrations of ammonia nitrogen in rumen fluid of sheep fed WCS diet ($P<0.01$). *In vivo* molar proportion of propionate was higher and the acetate to propionate ratio was lower for control diet than for WCS diet. It was concluded that feeding whole cottonseed reduced rumen fauna, ammonia nitrogen and the molar proportion of propionate and increased the acetate to propionate ratio, but had no effect on ruminal pH and total VFA concentration.

Key words: Sheep, Cottonseed, Volatile fatty acid, Protozoa, Ammonia nitrogen.

Abbreviations: WCS: whole cottonseed, VFA: volatile fatty acids, DMD: dry matter disappearance.

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تأثیر جیره دارای پنبه دانه کامل بر جمعیت پروتوزوآ و پارامترهای تخمیری در شکمبه گوسفند

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چکیده

آزمایش های *in vitro* و *in vivo* برای بررسی بازده پنبه دانه کامل بر جمعیت پروتوزوآ و پارامترهای تخمیری انجام شد. تیمارها عبارت بودند از: ۱) تیمار شاهد (بدون پنبه دانه کامل) و ۲) تیمار دارای ۲۰ درصد پنبه دانه کامل. درصد ناپدید شدن ماده خشک و خصوصیات تخمیری تیمارها با آزمایش *in vitro* تعیین شد. در آزمایش *in vivo*، مایع شکمبه از گوسفندان تغذیه شده با تیمارها با روش rumenocentesis (۳ ساعت بعد از تغذیه) در روزهای ۱، ۳، ۵، ۷، ۹، ۱۱، ۱۴، ۲۱ و ۲۸ جمع آوری شد. pH مایع شکمبه و شمارش پروتوزوآ برای هر یک از نمونه ها، و غلظت نیتروژن آمونیاکی و اسیدهای چرب فرار، تنها در نمونه های جمع آوری شده در روزهای ۷، ۱۴، ۲۱و ۲۸ تعیین شد. درصد ناپدید شدن ماده خشک در شرایط *in vitro* در ۷۲ ساعت پس از انکوباسیون برای تیمار شاهد نسبت به تیمار پنبه دانه کامل، بیشتر($P<۰/۰۱$) بود. غلظت کل اسیدهای چرب فرار در ۷۲ ساعت انکوباسیون برای تیمار شاهد نسبت به تیمار پنبه دانه کامل، بیشتر($P<۰/۰۵$) بود. در ۲۴ ساعت انکوباسیون، نسبت مولاری استات، پروپیونات، و والرات برای تیمار شاهد نسبت به تیمار پنبه دانه کامل بیشتر، اما نسبت مولاری ایزووالرات، کمتر ($P<۰/۰۵$) بود. در ۷۲ ساعت بعد از انکوباسیون نسبت استات به پروپیونات برای تیمار شاهد بیشتر ($P<۰/۰۵$) بود. تغذیه پنبه دانه کامل جمعیت کل پروتوزوآ را از تقریباً ۴۵۰۰۰۰ به ۲۴۰۰۰۰ در میلی لیتر کاهش($P<۰/۰۵$) داد و پروتوزوآهای holotrich و cellulolytic در نمونه های مایع شکمبه ناپدید شدند و تنها گونه *Entodinium* sp. باقی ماند. این تغییرات در جمعیت پروتوزوآ با غلظتهای کمتر نیتروژن آمونیاکی ($P<۰/۰۱$) در مایع شکمبه گوسفندان تغذیه شده با تیمار دارای پنبه دانه کامل، همراه بود. در شرایط *in vivo*، نسبت مولاری پروپیونات برای تیمار شاهد نسبت به تیمار پنبه دانه کامل بیشتر ولی نسبت استات به پروپیونات کمتر بود. نتایج این بررسی نشان داد که تغذیه پنبه دانه کامل، جمعیت پروتوزوآ، نیتروژن آمونیاکی و نسبت مولاری پروپیونات را کاهش و نسبت

استات به پروپیونات را افزایش داد ولی تأثیری بر pH مایع شکمبه و غلظت کل اسیدهای چرب فرار نداشت.

INTRODUCTION

The rumen has been the subject of many studies to understand the biological activities of different microorganisms and their relations. Williams and Coleman (47) showed different metabolic functions of protozoa present in the rumen; some may or may not be beneficial to the ruminal host. The ciliated protozoa consume and digest rumen bacteria, and increase recycling of microbial nitrogen in the rumen (18), resulting in a decreased amino acid supply to the intestine (43). Decrease in amino acid supply to the intestine is reported to be as high as 20 to 28 % (14). Consequently, the elimination of protozoa in the rumen is desirable when animal performance is limited by the availability of amino acids for absorption (19). In addition, ciliated protozoa contribute to ruminal production of methane (11) and associated loss of feed energy (3). Methanogens associated with ciliated protozoa are responsible for 9-25% of methanogenesis in ruminal fluid (31). Nitrogen and methane are both important environmental pollutants, and numerous studies have been done to manipulate the protozoal population in the rumen, including total defaunation. Some of the techniques, using chemical substances to remove protozoa from the rumen have been tested experimentally, but none of these techniques are practical, because of toxicity problems, either to the rumen bacteria or to the host animal (47). Recently, there has been an increased interest in plant secondary metabolites as possible defaunating agents, but there are presently no specific antiprotozoal agents commercially available. Different defaunation techniques have been reviewed recently (11) and it has been concluded that oil, milk fat (22, 24) and all of the unsaturated C18 fatty acids (29) are toxic to protozoa. Ivan *et al.* (16) reported that feeding sunflower oil (6% of dietary dry matter) decreased total protozoal numbers in rumen fluid samples from approximately 1,000,000 to less than 200,000 ml^{-1} within 6 days. Cottonseed oil is rich in linoleic acid (18:2) and can be used as a supplement to decrease protozoal population. However, using plant oil in ruminant diets is very expensive and not economically viable; an alternative approach is to utilize oilseeds such as whole cottonseed.

The objectives of the present study were to test the efficacy of dietary cottonseed to suppress protozoal numbers in the rumen and measure its effects on rumen fermentation

parameters. The effects of cottonseed on *in vitro* dry matter disappearance, kinetics of gas production and volatile fatty acid concentration were also studied.

MATERIALS AND METHODS

In Vitro Degradation

In vitro DM disappearance of the dried treatment diets was determined in five replications (five syringes, each ones as a replicate) after incubation with rumen fluid for 24 and 72 h using the Menke's gas test apparatus (27). Approximately 250-300 mg of dried sample was incubated with 30 ml buffer containing 10 ml rumen fluid in glass syringes. The rumen fluid was obtained from a ruminally cannulated cow fed the standard diet, *ad libitum*. The syringes and their contents were maintained at 39 °C in an incubator.

In vitro gas production was determined at 0.5, 2, 4, 8, 12, 24, 48 and 72 h after incubation of ground diets (1.0 mm screen) in rumen fluid in triplicates (26). Gas production data were fitted with the following equation (9) using non-linear regression of Statistical Analysis System (35):

$$P = a + b\,(1 - e^{-c(t-lt)})$$

where p represents the *in vitro* gas production (ml) at time t, $(a+b)$ is the potential gas production, c the fractional rate of gas production per hour and l_t represents the lag phase before gas production commenced.

Volatile fatty acids and lactic acid concentrations were determined in the supernatant from samples incubated with rumen fluid for 24 and 72 hours. After taking rumen fluid from syringes, the residue was filtered and dried at 55°C for 48 h to determine DM disappearance. Supernatant of incubated samples was collected into test tubes, deproteinized with 25% (wt/vol) metaphosphoric acid and then stored at -40°C until analyzed for VFA (27). After thawing at 4°C, the tubes were centrifuged at 12,000 rpm for 10 min, the supernatant was mixed with internal standard (0.3 ml 60 mM crotonic acid) and analyzed for VFA (column: 30m × 0.25mm id × 1.0 μ DB-FFAP fused silicon capillary, J. and W. Scientific Inc. Folsom, CA) by gas chromatography (Hewlett 5890 Packard Series Π).

In Vivo Study

Eight Iranian Naeini rams (over one year old) with an average body weight of 40 ± 5 kg were randomly divided into two groups and housed in individual stalls. Both groups were fed a control diet for 21 days prior to the start of the 28-day experimental period. Thereafter, one group (control) continued to receive the same diet while the other group received a diet containing 20% whole cottonseed (WCS). Mineral and vitamin additions were similar in both treatment diets. Ingredient composition of the experimental diets is given in Table 1. The sheep were fed TMR diets ad-libitum twice a day (800 and 1600h) and water was available at all times.

Rumen fluid was sampled during the experiments from each sheep at 1100 h on days 1, 3, 5, 7, 9, 11, 14, 21, and 28. The samples were taken through rumenocentesis (32), i.e., precutaneous needle aspiration from the caudoventral sac of the rumen. The area was clipped, scrubbed with povidone-iodine, and wiped with 70% ethanol. The area was sedated using 7-8 ml lidocain-epinephrine (1 + 0.008%). A stainless steel needle was passed through the skin and the abdominal muscles to the midline position 3-5 cm medially, and a 10-ml syringe was used to aspirate a minimum of 7.5 ml of fluid. After each sampling, a pen-strep 2 + 2 (Penicillin G procaine 2 MIU and Kanamycin sulfate eq. to 2 g base, NASR Pharmaceutical Co.) was injected intramuscularly for 2 days to prevent infection. The pH of rumen fluid was measured with a portable pH meter immediately after it was aspirated from the rumen. Ruminal volatile fatty acids (VFA) and ammonia N were determined in rumen fluid samples taken on days 7, 14, 21 and 28. A sub-sample (5 ml) of ruminal fluid was combined with 1 ml of 25% (wt./vol.) *meta*-phosphoric acid prior to freezing for analysis of VFA and ammonia N. Later the samples were thawed at 4°C and centrifuged at 12,000 rpm for 10 min. For VFA analysis, half of the supernatant was mixed with internal standard (0.3 ml of 60 mM crotonic acid) and analyzed (column: 25m×0.32m id×0.3 μ WCOT Fused Silica capillary) by gas chromatography (CHROMPACK, CP, 9002). Ammonia N was analyzed on the other half of the samples by the phenol-hypochlorite reaction (44).

Table 1. Ingredient and chemical composition of the diets (DM basis).

	Diet	
	Control	WCS
Ingredient (%)		
Alfalfa hay	14.00	19.33
Wheat straw	5.50	6.00
Corn silage	34.40	29.42
Barley grain, ground	8.50	8.00
Corn grain, ground	12.00	2.90
Cottonseed, whole with lint	0.00	20.00
Cottonseed meal	12.30	0.00
Soybean meal	10.30	9.52
Wheat bran	1.00	2.90
Dicalcium phosphate	0.60	0.53
Limestone	0.20	0.20
Sodium bicarbonate	0.30	0.30
Vitamin (A,D, and E) premix†	0.40	0.40
Trace-mineralized salt§	0.50	0.50
Chemical composition		
DM	48.75	47.00
Ether extract	3.15	6.50
CP	15.95	15.70
NDF	37.50	43.20
ADF	24.50	31.30
Lignin	4.52	8.60
NSC¶	39.90	30.30
Ash	3.25	4.30

WCS: Whole cottonseed.
† Contained 5,000,000 IU of Vitamin A; 5,000,000 IU of Vitamin D; and 500,000 IU of Vitamin E per Kg.
§ Composition: 75.15% NaCl, 20.5% Dynamad, 3.046% Mn, 1.025% Cu-sulfate, 0.253% Zn-sulfate, 0.015% EDDI-80, and 0.011% Na- selenide.
¶ NSC=non-structural carbohydrates, NSC= 100 - (CP + NDF+ EE +ash).

Ciliated protozoa were counted by a Neubauer improved bright-line counting cell (0.1 mm depth, Hausser Scientific, Horshman, PA, USA) in rumen fluid samples which were taken on days 1, 3, 5, 7, 9, 11, 14, 21 and 28 and preserved with methylgreen-formalin-saline (MFS) solution (33). Each sample was counted twice. During each counting, the numbers of different genera (7) in population were recorded and grouped as *Entodinium* sp., Holotrichs (*Isotricha* and *Dasytricha* sp.) and cellulolytic protozoa (*Polyplastron, Diplodinium* and *Enoploplastron* sp.).

Composite samples of each experimental diet were made at the time of mixing every day and dried by oven-drying at 55°C to a constant weight. Dried feed samples were ground to pass through a 1-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia) and were analyzed for NDF (41), ADF, lignin, and ash (2). The crude protein content of the diets and ingredients was determined by flash combustion (Carlo Erba Instruments, Milan, Italy).

Dietary ingredients and feed samples were analyzed for fatty acid content and composition (Table 2). To determine fatty acid composition, samples were dried and extracted in ethyl ether for six hours using the Goldfisch apparatus. Fat in feed samples was determined after evaporating the ethyl ether under nitrogen and the fat content was used for fatty acid analysis after methylation. The fatty acids were methylated by tetramethylguanidine (37). Briefly, about 10 to 20 mg of feed fat were accurately weighed, and 400 µl methanol and 100 µl tetramethylguanidine were added to the fat and placed in a boiling water-bath for 10 min. To this, 5 ml of saturated NaCl and 2 ml of petroleum ether were added, mixed for 10 min in a rocker mixer and centrifuged at 640 g for 10 min. The top petroleum ether layer was transferred to another clean tube and dried under nitrogen. Five ml of hexane was added, vortexed and a portion of solution (containing fatty acids) was transferred into a 2 ml gas chromatography vial that was capped and stored at -20 °C.

Fatty acid methylesters were quantified by gas chromatography (Hewlett 5890 Packard Series Π) with a flame ionization detector. Peak area was measured with a HP APG TOP integrator. Samples were injected through the HP autosampler (model 18596B) and HP 7673 injector (Hewlett-Packard Ltd. Mississauga, ON) onto a CP Sil 88 fused silica capillary column (60 m × 0.25mm, 0.25-mm file thickness; Agilent technologies, Mississauga, ON). Helium carrier gas flow was 1.7 ml min^{-1}, the injector temperature was set at 225°C and the detector was set at 300°C. The temperature program was as follows: level one: 50°C to 175°C at 25°C/min then held for 20 min, level two: 175°C to 225°C at 5°C/min then held for 15 min; run times were 51 min. The concentration of each fatty acid was calculated based on the relative area peak of standard fatty acids at their retention times.

Table 2. Fatty acid composition of diets and feed ingredients.

Fatty acid †	Diet		Corn silage	Cottonseed	Alfalfa	Wheat straw
	Control	WCS				
Total, % of DM	2.25	4.71	1.59	13.2	0.55	0.17
	(g 100g^{-1} of fatty acids)					
12:0	0.06	0.02	0.11	nd	0.13	0.03
13:0	nd §	nd	nd	nd	nd	nd
14:0	0.33	0.51	0.15	0.45	0.25	0.30
14:1(t9)	nd	nd	nd	nd	nd	nd
14:1(c9)	nd	nd	nd	nd	nd	nd
15:0	0.04	0.03	0.03	0.02	0.08	0.06
16:0	13.70	16.10	6.15	14.38	3.5	2.50
16:1(t9)	0.05	0.03	0.09	0.02	0.02	0.02
16:1(c9)	0.30	0.34	0.13	0.30	0.13	0.04
17:0	0.10	0.09	0.11	0.06	0.09	0.04
17:1(c9)	0.04	0.05	0.03	0.03	0.03	0.02
18:0	2.40	2.43	1.35	2.23	1.02	0.43
18:1(c9)	12.70	11.98	5.85	10.51	0.67	0.61
18:1(c11)	0.6	0.56	0.29	0.51	0.09	0.04
18:2(c9,c12)	35.72	38.20	16.76	45.30	4.34	1.74
18:3(c6,c9,c12)	0.01	nd	0.01	nd	0.02	0.45
18:3(c9,c12,c15)	4.48	1.30	8.28	0.13	7.50	0.15
20:0	0.30	0.23	0.22	0.18	0.26	0.03
20:1(c11)	0.16	0.08	0.07	0.05	0.02	0.01
20:5	0.32	0.17	0.01	0.12	0.32	0.27
22:0	nd	0.11	nd	nd	0.71	0.88
22:6	0.06	0.02	0.03	nd	nd	nd

WCS: Whole cottonseed.

† Expressed as number of carbons: number of double bonds.

§ nd = Not detectable.

Statistical Analysis

A single factor ANOVA was used to determine statistical differences between means for derived parameters *(a+b)*, *c* and L_t. The GLM procedure was used to determine statistical differences between treatment diets for *in vitro* parameters. The MIXED procedure was used to analyze the protozoa, pH, VFA and ammonia N data with diet × day of sampling and the diet × day of sampling interaction in the model. A split plot model, using days as the sub-plot, with an unstructured error matrix was used for all variables where convergence could be obtained; otherwise, a compound symmetry error structure was used. The Duncan's test was used to compare means for significance. All analyses were performed by using SAS (35).

RESULTS

In Vitro Dry Matter Disappearance and Fermentation Characteristics

The *in vitro* dry matter disappearances (DMD) at 24 h of incubation was similar between the two diets, but DMD at 72 h incubation was higher ($P<0.01$) for the control than for the WCS diet (Table 3). The lag time prior to initiation of gas production from the treatment diets during the *in vitro* gas production measurements was zero, indicating that there was no lag time. The differences between diets were not significant for fractional rate of gas production (c) or potential gas production ($a+b$) (Table 3).

The *in vitro* total VFA concentration (mM) at 24 h after incubation was similar between the two treatment diets, but the total VFA concentration (mM) at 72 h after incubation was lower ($P<0.05$) for the WCS diet than for the control diet (Table 4). After 24 h of incubation

Table 3. Dry matter disappearance (DMD) and the kinetics of *in vitro* gas production.

	Diet		SE (n=5)	P value
	Control	WCS		
DMD, % / 24 h after incubation	56.36	57.62	2.62	0.3570
DMD, % / 72 h after incubation	76.63	71.80	1.48	0.0013
In vitro gas production †				
$a + b$	22.99	21.48	0.90	0.7910
C	8.55	8.34	0.26	0.0920
L	0.00	0.00	------	------

WCS: Whole cottonseed.

† $a + b$ is potential gas production (ml 100 mg $^{-1}$ DM), c is rate constant of gas production (% h^{-1}) and l is lag time (h) prior to initiation of gas production in the equation $p = a + b\ (1 - e^{-c(t-lt)})$ (9).

the molar proportions (%) of acetate, propionate and valerate were higher ($P<0.05$) for the control diet, but the molar proportion (%) of isovalerate was lower ($P<0.05$) for the control diet. The acetate to propionate ratio was higher ($P<0.05$) for the control diet at 72 h after incubation (Table 4). The lactate and succinate concentrations were very low (0.02 mM) for treatment diets.

In Vivo Study

The protozoal population (number of total ciliated protozoa per ml fluid) in sheep on the treatment diets is shown in Fig. 1. The total protozoal population was different ($P<0.05$) between treatment diets on days 1, 5, 11 and 14. The protozoal population was decreased with

the WCS diet. The overall protozoal means during the experiment were 445,973 ± 34,767 ml^{-1} and 241,438 ± 28,362 ml^{-1} in control and WCS diets, respectively. The mean protozoal population on the first day of experiment was about 600,000 in control diet and 410,000 ml^{-1} in WCS diet. The population decreased to under 410,000 ml^{-1} in control diet and less than 250,000 ml^{-1} in WCS diet on day 3. From days 5 to 28, the population of protozoa for the control diet ranged between approximately 350,000 and 580,000 ml^{-1}. In contrast to the control diet, the protozoa population on the WCS diet and then recovered on day 28, when it reached 300,000 ml^{-1}. These data show that oil in cottonseed decreased the protozoal population from 450,000 to 240,000 ml^{-1}.

Table 4. Volatile fatty acid content (% of molar proportions) and total VFA (mM) of *in vitro* for 24 and 72 h incubation of diets.

Incubation Time (h)	VFA	Diet		SE (n=5)	P value
		Control	WCS		
24	Total VFA	121.9	120.6	0.711	0.3099
	Acetate	56.4	56.2	0.078	0.0021
	Propionate	24.4	23.1	0.289	0.0206
	Isobutyrate	1.2	1.3	0.018	0.9676
	Butyrate	12.8	12.4	0.285	0.3200
	Isovalerate	2.36	2.45	0.025	0.0344
	Valerate	3.3	2.7	0.083	0.0001
	Caproate	0.4	0.4	0.004	0.5317
	Acetate/propionate	2.3	2.4	0.027	0.1618
72	Total VFA	150.1	134.1	2.340	0.0405
	Acetate	55.9	56.8	0.210	0.2452
	Propionate	22.2	22.9	0.140	0.1872
	Isobutyrate	1.7	1.7	0.008	0.4162
	Butyrate	11.9	11.6	0.122	0.0910
	Isovalerate	3.9	3.7	0.051	0.1233
	Valerate	3.1	2.9	0.041	0.1261
	Caproate	0.5	0.4	0.008	0.2831
	Acetate/propionate	2.52	2.48	0.007	0.0228

WCS: Whole cottonseed.

The percentage of *Entodinium* sp., Holotrichs (*Isotricha* and *Dasytricha* sp.) and cellulolytic (*Polyplastron*, *Diplodinium* and *enoploplastron* sp.) protozoa, relative to total protozoa count in the control diet, ranged between 93.2 and 96, 2.5 and 3.6 and 1.5 and 4 %, respectively. Holotrich protozoa virtually disappeared from the rumen of sheep on the WCS

diet after 14 days. Similarly, the cellulolytic protozoa disappeared from the rumen of sheep on the WCS diet on day 28. Also, in sheep fed WCS diet both the Holotrich and cellulolytic protozoa population in rumen fluid of sheep were decreased after day 3 of the experiment (Table 5).

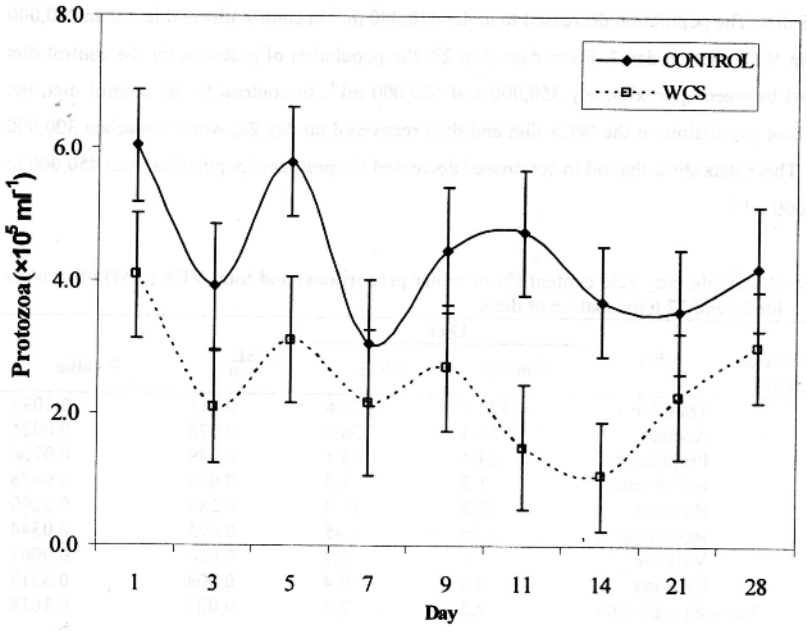

Fig. 1. Effects of treatment diets on ciliate protozoal concentrations in ruminal fluid of sheep. Vertical bars are standard error (n=4). Differences between treatment means were significant (P< 0.05) on days 1, 5, 11, and 14 only.

Mean pH values of the ruminal fluid during the experiment are shown in Fig 2. There was not a treatment × day sampling interaction (P=0.69). The mean pH was similar for control diet and WCS diet, while it was significantly higher for the control diet than for the WCS diet on day 5 only. Generally, pH increased for control and WCS diets during the first 3 days of the experiment, from 6.3 and 6.1 to about 6.6 and 6.8, respectively. The pH was then increased to 6.7 for control diet and declined to 6.3 for WCS diet by day 5. From days 7 to 28, the mean pH for the control diet ranged between 6.2 and 6.6, while for the WCS diet it ranged between 6.4 and 6.5. The overall means during the experiment were 6.48 ± 0.06 and 6.42 ± 0.05 for the control and WCS diets, respectively (Table 6).

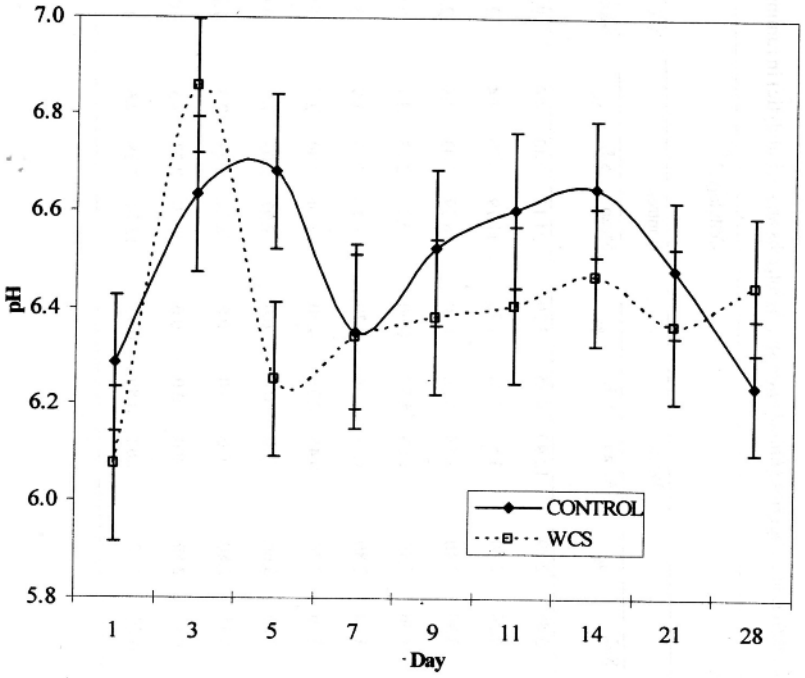

Fig. 2. Effect of treatment diets on pH in ruminal fluid of sheep. Vertical bars are standard error ($n=4$). The differences between treatment means were significant ($P< 0.05$) on day 5 only.

Table 5. Effect of diets on the concentration ($\times 10^3$ ml^{-1}) of ciliated protozoa of different categories (*Entodinium* sp., Holotrichs and cellulolytic) in ruminal fluid of sheep.

Day	*Entodinium sp.*						Holotrichs						Cellulolytic¶					
	Control			WCS			Control			WCS			Control			WCS		
	Mean	S.E.†	%§	Mean	S.E.	%	Mean	S.E.	%	Mean	S.E.	%	Mean	S.E.	%	Mean	S.E.	%
1	562.04	82.56	93.2	387.51	94.04	94.3	16.89	3.80	2.80	12.99	4.35	3.20	24.12	2.40	4.0	9.69	2.73	2.50
3	382.32	94.24	95.0	204.22	79.53	97.2	10.20	4.36	2.60	3.57	3.80	1.70	11.79	3.30	2.4	2.31	2.40	1.10
5	544.40	82.56	94.0	283.99	90.57	98.0	17.95	3.80	3.10	3.58	4.36	1.20	16.79	2.03	2.9	2.46	2.30	0.80
7	297.25	94.25	93.5	187.19	109.0	98.2	10.71	4.36	3.40	1.66	5.29	0.90	9.96	2.73	3.1	0.82	2.75	0.90
9	448.89	90.58	95.3	244.70	90.57	98.3	12.85	4.36	2.80	1.74	4.36	0.70	9.16	2.73	1.9	2.43	2.30	1.00
11	455.06	94.24	94.1	127.42	90.57	98.7	15.47	3.36	3.20	0.45	4.36	0.40	13.44	2.30	2.7	1.08	2.30	0.90
14	346.06	82.56	93.7	105.85	79.53	99.2	13.30	3.80	3.60	0.0	0.0	0.0	9.97	2.03	2.7	0.85	2.03	0.80
21	345.69	94.07	94.9	230.86	94.00	99.6	11.04	4.35	2.80	0.0	0.0	0.0	8.54	2.29	2.3	1.11	2.72	0.40
28	503.11	90.41	96.0	301.40	82.56	100.0	11.62	4.31	2.50	0.0	0.0	0.0	6.70	2.73	1.5	0.0	0.0	0.0
Mean	420.45	32.67	94.4	236.43	27.54	98.2	22.44	6.20	2.98	2.62	0.80	0.97	12.24	1.28	2.6	4.71	1.56	0.93

WCS: Whole cottonseed.

† Standard error (n=4).

§ Percent of total protozoa count.

¶ *Polyplastron, Diplodinium* and *Enoplastron* sp.

The mean concentration of ammonia N is given in Table 6. The treatment × day of sampling interaction was not significant (P=0.72). The ammonia nitrogen concentration in the ruminal fluid of sheep fed with WCS diet was lower (P<0.01) than the control diet. The differences between the control diet and WCS diet were significant (P<0.05) on day 28. The concentration of ammonia N increased during the experiment in sheep fed the control diet, while on the WCS diet, the concentration increased between days 7 to 21 and then decreased on day 28 (Fig. 3).

Total VFA concentration (mM) was numerically higher with the control diet that with the WCS diet, although not statistically different (Table 6). The molar proportion (%) of

Table 6. Effect of diets on ammonia nitrogen (NH_3N), pH and volatile fatty acids (VFA) concentration and molar proportion in ruminal fluid of sheep†.

	Diet				Effects		
	Control		WCS		Diet	Day	Diet × day
	Mean	S.E	Mean	S.E.			
NH_3N (mg 100 ml^{-1})	32.25	4.62	20.72	2.27	*	*	N.S.§
pH	6.48	0.06	6.42	0.05	N.S.	*	N.S.
Total VFA (mmol l^{-1})	97.17	20.80	90.08	30.39	N.S.	N.S.	N.S.
Molar proportion (%)							
Acetate	70.95	1.52	70.00	1.20	N.S.	N.S.	N.S.
Propionate	19.09	1.21	18.00	1.66	*	N.S.	N.S.
Isobutyrate	0.61	0.12	0.57	0.15	N.S.	N.S.	N.S.
Butyrate	8.22	1.74	9.50	1.23	N.S.	N.S.	N.S.
Isovalerate	0.57	0.19	0.55	0.09	N.S.	N.S.	N.S.
Valerate	0.76	0.14	0.67	0.11	N.S.	N.S.	N.S.
Caproate	0.41	0.20	0.44	0.20	N.S.	N.S.	N.S.
Acetate/propionate	3.73	0.25	3.95	0.45	*	N.S.	N.S.

WCS: Whole cottonseed.

† Means and standard errors (S.E.) of four sheep and four sampling days; 7, 14, 21, and 28.

§ Not significant ($P > 0.05$).

* $P < 0.05$.

** $P < 0.01$.

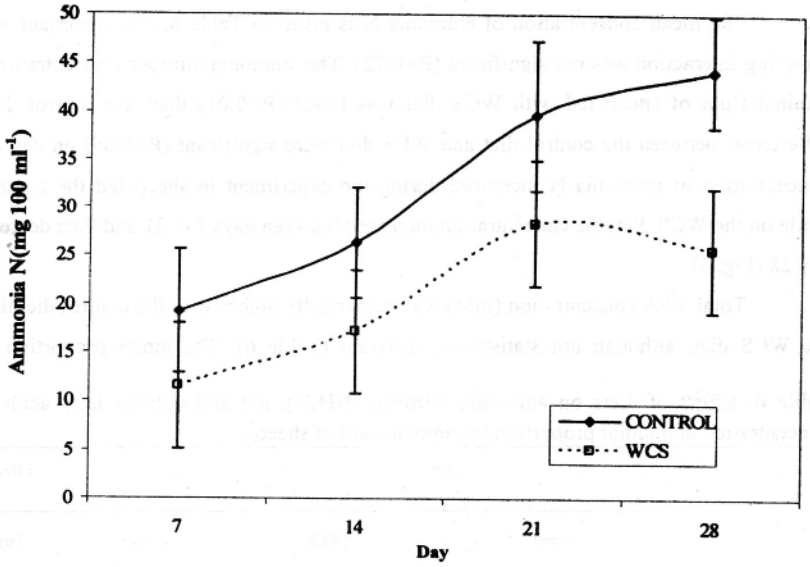

Fig. 3. Effect of treatment diets on the concentration of ammonia nitrogen in ruminal fluid of sheep. Vertical bars are standard error ($n=4$). The differences between treatment means were significant ($P< 0.05$) on day 28 only.

propionate was higher (P<0.05), and the acetate to propionate ratio was lower (P<0.05) for the control diet compared to the WCS diet. There was no significant effect of the day of sampling and diet × day of sampling interaction (P>0.05) on each VFA.

DISCUSSION

In vitro dry matter disappearance (DMD) was affected by whole cottonseed supplement after 72 h incubation. Devendra and Lewis (8) reported four possible mechanisms through which oils rich in polyunsaturated fatty acids could depress cell-wall degradation: physical coating of fiber by lipids, shortage of cations (e.g. calcium) due to formation of insoluble soaps, inhibition of rumen microbial activity, and modification of the microbial population because fatty acids are toxic to bacteria. This reduction in DMD could be due to cottonseed oil that is rich in polyunsaturated fatty acids. The *in vitro* total VFA concentration (mM) at 72 h after incubation with the WCS diet was lower than with the control diet. These

results confirmed the study of Chalupa *et al.* (6) who found in an *in vitro* study that addition of free $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ to a hay-grain diet decreased the VFA concentrations and acetate-to-propionate ratio. In some experiments, WCS had no effect on rumen fermentation (4, 50, 10). In the *in vivo* study, whole cottonseed reduced the molar proportion of propionate without any effect on butyrate. In contrast with the present results, Wettstein *et al.* (45) reported that by using canola oil, the molar proportion of propionate was increased but that of butyrate decreased. Mohamed *et al.* (28) and Keele *et al.* (21) reported that WCS feeding decreased the butyrate concentration. After 24 h incubation, whole cottonseed caused reduction of molar proportion of acetate and this might be a direct effect of the inclusion of unsaturated fatty acid on microorganisms, because WCS diet contained more unsaturated fatty acid ($C_{18:2}$) than the control diet. Machmueller *et al.* (25) reported that lipid supplementation (oilseeds) depressed the concentration of acetate and butyrate. The acetate to propionate ratio was lower for the WCS diet at 72 h after incubation. In some reports, the acetate to propionate ratio increased with WCS (12, 38), whereas in others it decreased (28, 21, 1). These results support the suggestion of Kajikawa *et al.* (20) that the effect of feeding WCS on rumen fermentation depends on the basal fermentation balance.

The whole cottonseed diet also decreased the protozoal population by approximately 50%. In some studies, ruminal protozoa were reduced by WCS (12, 28). It has been reported that oils (24) and unsaturated C_{18} fatty acids (29) are toxic for protozoa. Williams (46) reported that protozoa have a limited ability to absorb and transform lipids and high dietary lipid concentrations are toxic to them. Several studies (5, 13, 39) suggested that fat and oil supplements decreased protozoal population. Likewise, Ivan *et al.* (16) reported that feeding sunflower oil decreased the total protozoa numbers from approximately 1,000,000 to less than 200,000 ml^{-1} and eliminated the Holotrich and cellulolytic protozoa from the rumen. In agreement with these results, Holotrich and cellulolytic protozoa virtually disappeared from the rumen of sheep within 14 and 28 days on the WCS diet, respectively. After 14 days, the only protozoa present in the rumen of the cottonseed-fed sheep were *Entodinium* sp. Ivan *et al.* (15, 17) reported that Entodinium might be detrimental to the protein nutrition of the ruminant host. Ivan *et al.* (16) found that the Holotrichs protozoa were the most susceptible group to the toxic effects of some fatty acids such as linoleic acid, followed by cellulolytic protozoa. In the present study, WCS diet had more linoleic acid than the control diet and the present results agree with those of Ivan *et al.* (16). In the present study, partial defaunation was achieved with a

WCS diet containing 20% whole cottonseed. The level of incorporation of WCS as well as the duration of feeding to achieve complete defaunation still needs to be determined.

In this study, rumen ammonia N decreased with the WCS diet. Defaunation is known to decrease ammonia N concentration. Veira *et al.* (42) found that defaunation is associated with decreased rumen ammonia concentration and increased amino acid flow from the rumen to the intestinal tract for absorption and utilization by the host. Reduction in rumen ammonia N might be related to two reasons: 1) defaunation decreased N recycling between bacteria, protozoa and ammonia pools resulting from engulfment and digestion of bacteria by protozoa (23), 2) defaunation results in increased rumen bacteria numbers which use ammonia nitrogen as a substrate for cell synthesis and subsequently there is an increased demand for ammonia (36). Tagari *et al.* (40) reported that high rumen degradability of WCS protein was reflected in increased rumen ammonia *in vitro*. In some experiments, where WCS supplied up to 200 g/kg of the dietary DM (28, 34), rumen ammonia level was not modified by WCS inclusion. Defaunation is also associated with lower and more variable rumen pH (42). Therefore, protozoa exert a stabilizing effect on rumen pH, which is probably due to their uptake and removal of starch from immediate bacterial fermentation (48). Secondly, the faster clearance of exogenous lactate as well as lower levels of endogenously produced lactate in faunated compared with ciliate-free sheep also contribute to this stabilizing effect (30). In the present study, however, mean pH value was similar between the two diets, except on day 5.

In the present study, the total VFA and molar proportion of each VFA was not affected by whole cottonseed supplement, but the molar proportion of propionate decreased and the acetate to propionate ratio increased. Wettstein *et al.* (45) and Ivan *et al.* (16) reported that using oil supplement decreased total VFA concentration, while the molar proportion of propionate was increased and that of butyrate decreased; Machmueller *et al.* (25) found that lipid supplementation (oilseeds) depressed the concentration of acetate and butyrate. The present results about VFA parameters might be due to 1) low oil supplement in WCS diet, 2) cottonseed fat may be released slowly in the rumen, 3) WCS leaves the rumen still partially enclosed within the seed, or that 4) the large errors arising from large between animal variation obscured the reduction in total VFA concentration that occurred.

CONCLUSIONS

Feeding whole cottonseed reduced rumen fauna, ammonia nitrogen and the molar proportion of propionate and increased the acetate to propionate ratio, but had no effect on

ruminal pH and total VFA concentration. The results of the *in vitro* study revealed that at 24 h of incubation the molar proportion (%) of acetate, propionate and valerate was higher, but the molar proportion of isovalerate was lower for the control diet than for the WCS diet. Finally, these results suggest that strategies, such as, inclusion of whole cottonseed may be effective in decreasing protozoal population in the rumen. More research is needed in order to investigate the period and percentage of using this material for complete defaunation.

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