Available online at www.sciencedirect.com
SCIENCE DIRECT®
Animal Feed Science and Technology
105 (2003) 163–184
ANIMAL FEED SCIENCE AND TECHNOLOGY
www.elsevier.com/locate/anifeedsci

Evaluation of several potential bioactive agents for reducing protozoal activity in vitro

A.N. Hristov [a], M. Ivan [b], L. Neill [b], T.A. McAllister [b,*]

[a] *Department of Animal and Veterinary Science, University of Idaho, Moscow, ID 83844-2330, USA*
[b] *Agriculture and Agri-Food Canada, Lethbridge Research Centre, P.O. Box 3000, Lethbridge, AB, Canada T1J 4B1*

Received 29 April 2002; received in revised form 23 December 2002; accepted 9 January 2003

Abstract

The effects of 14 bioactive agents on ruminal fermentation and protozoal activity were investigated in vitro as potential feed additives to improve feed efficiency. Agents studied were: lecithin, herring meal, canola oil, coconut oil, linseed oil, palm oil, and soybean oil, with each oil at final concentrations of 0.5, 1.0 and 2.0%; the saponin-containing plants *Yucca schidigera* (yucca) and *Quillaja saponaria* Molina (quillaja), tannic acid, and bentonite, each at 0.1, 0.2, and 0.4%; the surfactant Tween 80 (at 0.05, 0.1, and 0.2%); and the ionophores monensin (at 2.5, 5.0, and 10 mg/l) and salinomycin (at 1.25, 2.5, and 5.0 mg/l). Duplicate 4 h incubations each included controls (no additives) and ^{15}N labeled casein as a N tracer. Yucca, tannic acid and both ionophores decreased ($P < 0.05$) NH_3 concentrations. Total free amino acids (TFAA) were decreased ($P < 0.05$) by yucca and by bentonite, but were increased ($P < 0.05$) by fish meal, monensin and salinomycin. Tannic acid and bentonite reduced ($P < 0.05$) total VFA concentrations, and tannic acid reduced ($P < 0.05$) the acetate:propionate (A:P) ratio. Bentonite, tannic acid, palm oil, quillaja and salinomycin reduced ($P < 0.05$) carboxymethylcellulase (CMCase), xylanase, and amylase activities, whereas monensin, lecithin, Tween 80, yucca, and the feed oils (except coconut) decreased ($P < 0.05$) amylase activity only. Protozoal populations, which comprised 84% *Entodinium* spp., decreased ($P < 0.05$) with tannic acid, lecithin and quillaja. Tannic acid, fish meal, and the ionophores reduced ($P < 0.05$) the rate of protozoal engulfment of bacteria. Fish meal and salinomycin, and the higher concentrations of quillaja and Tween 80 depressed ($P < 0.05$) incorporation of ^{15}N from

Abbreviations: BW, body weight; CMCase, carboxymethylcellulase; CT, condensed tannins; DM, dry matter; HT, hydrolyzable tannins; MMB, modified McDougall's buffer; S.E., standard error; TFAA, total free amino acids; VFA, volatile fatty acids

* Corresponding author. Tel.: +1-403-317-2240; fax: +1-403-382-3156.
E-mail address: mcallister@agr.gc.ca (T.A. McAllister).

doi:10.1016/S0377-8401(03)00060-9

casein into bacterial cells. Only lecithin effectively reduced protozoal numbers, by 27%, without impeding the extent of ^{15}N incorporation into bacteria.

Keywords: Feed; Ionophores; Oils; Protozoa; Ruminal fermentation; Saponins

1. Introduction

Ciliate protozoa are believed to contribute significantly to intraruminal cycling of microbial N and to decrease the efficiency of microbial protein synthesis (Jouany, 1996), thus reducing protozoal populations could improve dietary N utilization and increase microbial protein flow to the intestine (Williams and Coleman, 1992). We have recently documented maintenance of a significant protozoal population, comprising almost entirely non-cellulolytic species, in beef cattle fed high grain barley-based finishing diets (Hristov et al., 2001). The opportunity exists, therefore, to improve nutrient utilization by these feedlot cattle through protozoal control. Reducing the protozoal population could offer the benefits of enhancing daily gain and improving feed efficiency of barley-fed cattle without adversely affecting ruminal organic matter degradation.

Dietary fats have been shown to generally inhibit microbial activities and digestion in the rumen (Jenkins, 1993); oils rich in saturated medium-chain and unsaturated long-chain fatty acids have been shown to inhibit ruminal protozoa in vitro and in vivo (Newbold and Chamberlain, 1988; Machmüller and Kreuzer, 1999; Ivan et al., 2001). Other bioactive agents that have potential to inhibit ruminal ciliates include ionophore antibiotics (Kobayashi et al., 1990; Williams and Coleman, 1992), steroidal saponins (Hristov et al., 1999a), condensed tannins (Wang et al., 1996), surface-active agents (Burggraaf and Leng, 1980), and bentonites (Wallace and Newbold, 1991; Ivan et al., 1992). However, some of these agents also exert deleterious effects on ruminal bacteria, such as reduced cellulolytic bacterial populations and decreased microbial N flow to abomasum/duodenum in association with ionophores (Kobayashi et al., 1990). Thus, any benefit in ruminal protein metabolism arising from reduced protozoal numbers may be negated by a reduction in bacterial growth and nutrient digestion. Consequently, the antiprotozoal properties of these compounds should be studied in conjunction with their effects on ruminal fermentation, to ensure that their inclusion in the diet will have a net benefit on ruminal metabolism.

The objective of this study was to investigate several bioactive agents with demonstrated antiprotozoal activities for their effects on fermentation, protozoal numbers, and protozoal and bacterial activities in ruminal fluid from cattle fed a high-energy, barley grain-based finishing diet.

2. Materials and methods

2.1. Ruminal inocula

Two ruminally cannulated Hereford heifers (503 ± 3.9 kg BW) with ad libitum access to a diet composed of (DM basis) 90% steam-rolled barley grain, 4% barley silage, 5%

soybean meal, and 1% mineralized salt were used as donors of ruminal content. Their feed was delivered at 9:00 and 16:00 h daily in quantities sufficient to ensure 5% orts, and they were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 1993).

For each of four incubations, ruminal contents were collected from the ventral sac, reticulum, and feed mat of the rumen of each heifer, 2 h after the morning feeding. In preparing inoculum, as much plant material was removed from the ruminal contents as possible in order to minimize non-microbial N contamination of the samples processed subsequently for ^{15}N-enrichment determinations. Whole contents from both heifers were combined and squeezed through two layers of cheesecloth. Solids retained on the cheesecloth were resuspended in a volume of warm (39 °C) buffer equal to the volume of filtrate. The buffer (pH 6.8) was that described by McDougall (1948), pre-gassed with CO_2 and amended (per liter) with 5 g DL-glucose and 0.5 g L-cysteine HCl (Sigma Chemical Co., St. Louis, MO, USA), and was designated as modified McDougall's buffer (MMB). The suspension was agitated vigorously to detach loosely feed-adherent microorganisms (Hristov and Broderick, 1996) and squeezed again through two layers of cheesecloth. The two filtrates were combined and the inoculum was transported immediately to the laboratory in an insulated container. Feed particles were partitioned by flotation (Hristov and Broderick, 1994) and discarded. The remaining inoculum was used in the in vitro incubations.

2.2. In vitro incubations

Four separate 4 h incubations allowed each of 14 bioactive agents to be incubated twice, at three levels of inclusion (Table 1). A short-term in vitro incubation was selected in order to mimic the rumen microbial environment as closely as possible (Hristov and Broderick, 1994), and to improve the reliability of the estimate of fermentation parameters in the rumen as described by Hungate (1966), in reference to Markoff (1913). The 14 agents were studied in two groups with one group included in the first two incubations (Exp. 1) and the second group in the third and fourth incubations (Exp. 2). For each incubation, the selected agents were pre-measured into 450 ml culture bottles prior to preparation of the inoculum. Concentrations selected for the study were based on commonly fed dietary levels (feed oils, fish meal, lecithin), manufacturers' recommended doses (ionophores), and results of previous research with the other agents (Abdullah et al., 1995; Hristov et al., 1999b; Muhammed et al., 1994). Duplicate bottles were prepared for each treatment × level combination, and three control bottles with no bioactive agents added were also included in each incubation.

^{15}Nitrogen-labeled casein (Hristov et al., 2002) was used as a tracer. The ^{15}N-casein contained 16.0% N (S.E. = 0.22) and 0.1977 at.% excess ^{15}N (S.E. = 0.00020) and was dosed at 500 mg/bottle. To the pre-measured test compounds in each bottle were added: 5 ml of a 10% (w/v) solution of ^{15}N-casein in 0.1N NaOH, 65 ml of warmed, CO_2-gassed MMB, and 140 ml of ruminal inoculum. The bottles were flushed with CO_2, capped and affixed to a rocking platform for incubation at 39 °C for 4 h.

At the end of the incubation, each bottle was inserted into crushed ice. The pH of the incubation mixture was recorded, and subsamples were taken for protozoal counts, isolation of bacterial and protozoal pellets, and measurement of carboxymethylcellulase (CMCase),

Table 1
Bioactive agents incubated in vitro with ruminal inoculum[a]

Agent	Treatment designation	Levels of inclusion[b]	Exp. no.	Source and notes
Dietary oils (%, v/v)				
Canola	CAN	0.5, 1.0, 2.0	1	Canbra Foods Ltd., Lethbridge, AB, Canada
Coconut	COCO	0.5, 1.0, 2.0	1	Hanif's International Foods Ltd., N. Delta, BC, Canada
Linseed	LIN	0.5, 1.0, 2.0	1	Purified Boiled, Recochem Inc., Edmonton, AB, Canada
Palm	PALM	0.5, 1.0, 2.0	2	African Palm Oil, Hanif's International Foods
Soybean	SOY	0.5, 1.0, 2.0	2	100% Expeller Pressed, Hain Pure Foods Co. Inc., Los Angeles, CA, USA
Ionophores (μg/ml)				
Monensin	MON	2.5, 5.0, 10.0	1	Elanco Animal Health, Guelph, ON, Canada
Salinomycin	SAL	1.25, 2.5, 5.0	2	Pfizer Animal Health Division Orangeville, ON, Canada
Saponin-containing plants[c]				
Yucca schidigera	YUCC	0.1, 0.2, 0.4	1	Desert King International, San Diego, CA
Quillaja saponaria	QUIL	0.1, 0.2, 0.4	2	Desert King International, San Diego, CA
Surfactant (%, (v/v))				
Tween 80	T80	0.05, 0.1, 0.2	2	Sigma Chemical Co., St. Louis, MO, USA
Others (%, (w/v))				
Fish (herring) meal[d]	FISH	0.5, 1.0, 2.0	2	West Coast Reduction, Vancouver, BC, Canada
Lecithin	LECI	0.5, 1.0, 2.0	2	Liquid Soya Lecithin, Fearn Natural Foods, Mequon, WI, USA
Bentonite	BENT	0.1, 0.2, 0.4	1	Purified grade powder, B235, Fisher Scientific Limited, Nepean, ON, Canada
Tannic acid	TANN	0.1, 0.2, 0.4	1	ACS Reagent Grade, Sigma Chemical Co.

[a] Four 4 h incubations were conducted. In two groups, the bioactive agents were each incubated twice, with treatment × level duplicated within incubation ($n = 4$). Exps. 1 and 2 refer to the incubation groups.

[b] Final concentrations in the 210 ml incubations. In subsequent tables, these levels are denoted L1–L3, respectively.

[c] *Yucca schidigera* was included (%, (w/v)) as a powdered whole-plant preparation (DK Sarsaponin 30) containing 4.4% (w/w) saponins (Wang et al., 1998); *Quillaja saponaria* was included (% (w/v)) as a powdered extract of the Chilian tree, *Q. saponaria* Molina containing 10% (w/w) saponins (Desert King International).

[d] Fish meal (93% DM) contained (DM basis) 72% crude protein, 7.6% crude fat and 1% fiber.

xylanase, amylase and deaminative activities and concentrations of reducing sugars, ammonia (NH_3), total free amino acids (TFAA), soluble protein, volatile fatty acids (VFA), and lactate.

2.3. Sample analyses

Samples for protozoal counts were preserved with 0.2 volumes of methyl green:formalin: saline solution (Ogimoto and Imai, 1981). Aliquots for preparation of protozoal pellets were preserved with formalin (5% (v/v), final concentration), and centrifuged at 400 ×

g for 5 min at 4 °C. The resulting pellets were washed three times with formol–saline solution (10% (v/v) formol in 0.9% (w/v) aqueous NaCl) and re-centrifuged under the same conditions. The pellets, presumed to comprise primarily protozoal cells, were freeze-dried and analyzed for ^{15}N-enrichment of the total N. Bacterial pellets were isolated by differential centrifugation where the subsamples were centrifuged at 400 × g (5 min; 4 °C), and the supernatant was preserved with 5% (v/v) saturated $HgCl_2$, then centrifuged at 28,000 × g (20 min; 4 °C). The pellets were freeze-dried and analyzed for ^{15}N-enrichment of the total N.

Total N and ^{15}N-enrichment of the bacterial and protozoal pellets were determined on a nitrogen analyzer (Model 1500, Carlo Erba Instruments, Milan, Italy) equipped with a mass-ratio spectrometer (Optima^{TM}, VG Instruments, Middlewich, UK). Ciliate protozoal counts and analyses for enzymatic activities and the fermentation parameters listed above were conducted as described by Hristov et al. (2001).

Protozoal activity was determined on the basis of ^{15}N tracer found in the protozoal pellets, which was presumed to be of predominantly bacterial origin. The proportion of protozoal protein originating from bacterial protein (%) was calculated as:

$$\frac{^{15}N_{protozoa}}{^{15}N_{bacteria}} \times 100\% \quad (1)$$

where $^{15}N_{protozoa}$ is the ^{15}N-enrichment of protozoal pellets (at.% excess) at 4 h, and $^{15}N_{bacteria}$ is the ^{15}N-enrichment of bacterial pellets (at.% excess) at 4 h.

2.4. Statistical analyses

Experimental data were analyzed separately for Exps. 1 and 2 using the GLM procedure of the SAS Institute (SAS, 1996) as a completely randomized design with treatments in a 7 × 3 factorial arrangement plus a control carried twice (for each incubation), each with two replicates. When treatment × level interaction was significant, data were analyzed within application level. Treatment means within or across application levels were separated by pair wise t-test. All statistical computations were carried out using SAS (1996).

3. Results

Measurements of ruminal fermentation parameters in the incubation bottles from the two incubation groups (i.e. Exps. 1 and 2) are in Tables 2 and 3, respectively. Compared with the respective controls, pH after 4 h of incubation decreased ($P < 0.05$) with canola oil and linseed oil, and increased ($P < 0.05$) with the highest level of salinomycin.

Ammonia concentrations in ruminal inocula prepared for Exp. 1 were lower ($P < 0.05$) than those in the inocula prepared for Exp. 2 (data not shown). Compared with the respective controls, ammonia concentration was reduced ($P < 0.05$) by bentonite, monensin, yucca (excepting the lowest level), and particularly tannic acid in the first incubation group (Table 2), and by salinomycin in the second group (Table 3). In Exp. 2, fish meal and Tween 80 increased ($P < 0.05$) the 4 h ammonia concentration.

Table 2
Effects of oils and bioactive agents on fermentation characteristics of in vitro ruminal incubations (Exp. 1)

Parameter[c]	Level[d]	Control	Treatment[a]							S.E.	Level of significance[b]		
			CAN	COCO	LIN	MON	YUCC	BENT	TANN		T	L	T × L
pH		6.68	6.49[e]	6.60	6.46[e]	6.79	6.56	6.56	6.59	0.021	***	NS	NS
Ammonia (mmol/l)	L1	16.4	16.9	16.5	17.0	13.6[e]	15.5	14.8[e]	11.9[e]	0.48	***	***	***
	L2		16.4	16.4	16.6	15.2[e]	13.9[e]	15.0[e]	8.8[e]				
	L3		16.6	16.5	16.4	14.1[e]	11.9[e]	14.5[e]	5.6[e]				
TFAA (mmol/l)		5.7	4.9[e]	5.7	5.2	8.0[e]	4.7[e]	4.4[e]	5.9	0.16	***	NS	NS
RS (mmol/l)	L1	0.47	0.46	0.44	0.51	0.58	0.34	0.57	2.96[e]	0.076	***	***	***
	L2		0.46	0.39	0.41	0.55	0.36	0.38	3.85[e]				
	L3		0.43	0.23[e]	0.39	0.56	0.36	0.35	4.96[e]				
Soluble protein (g/l)	L1	4.8	4.7	4.9	5.5[e]	4.9	4.6	4.0[e]	5.4[f]	0.27	***	NS	*
	L2		4.8	4.8	5.0	5.1	4.6	3.5[e]	6.5[f]				
	L3		4.8	4.8	5.4[e]	4.9	4.6	3.2[e]	7.1[e]				
VFA (mmol/l)													
Acetic		74.4	75.8	76.3	73.5	74.7	73.1	67.8	63.8[e]	1.25	***	NS	NS
Propionic		35.4	35.1	35.6	34.7	35.4	35.5	33.1[e]	32.9[e]	0.28	*	NS	NS
Isobutyric	L1	1.26	1.23	1.34[e]	1.27	1.09[e]	1.27	1.17[e]	1.07[e]	0.033	***	NS	**
	L2		1.24	1.30	1.25	1.22	1.20[e]	1.25	1.04[e]				
	L3		1.28	1.29	1.32	1.17[e]	1.13[e]	1.18[e]	0.9[e]				
Butyric	L1	11.0	10.9	12.9[e]	11.2	9.9[e]	11.1	10.3	10.3[e]	0.35	***	NS	*
	L2		10.9	11.6	11.6	11.2	10.7	11.2	10.4				
	L3		11.3	11.5	11.6	10.8	10.4	10.6	9.5[e]				
Isovaleric	L1	2.43	2.38	2.57	2.50	2.00[e]	2.48	2.30	2.06[e]	0.075	***	NS	**
	L2		2.38	2.52	2.50	2.34	2.35	2.48	1.95[e]				
	L3		2.57	2.50	2.66[e]	2.20[e]	2.25[e]	2.33	1.68[e]				

Valeric	L1	2.80	2.80	2.94	2.92	1.86[e]	2.90	2.70	2.56[e]	0.092	***	NS	***
	L2		2.75	2.90	2.95	2.53[e]	2.86	2.97	2.35[e]				
	L3		2.95	2.81	3.06[e]	2.23[e]	2.79	2.81	1.86[e]				
Total VFA (mmol/l)		127.3	128.5	130.5	126.2	126.4	125.8	118.0[e]	112.0[e]	1.49	**	NS	NS
A:P		2.13	2.18	2.15	2.12	2.12	2.07	2.06	1.94[e]	0.038	**	NS	NS
Lactate (mmol/l)		0.17	0.25	0.16	0.23	0.20	0.37	0.16	NP[g]	0.036	NS	*	NS

NS, not significant ($P > 0.05$).

[a] Abbreviations are defined in Table 1.

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] TFAA, total free amino acids; RS, reducing sugars; VFA, volatile fatty acids.

[d] Levels L1–L3 refer to inclusion of CAN, COCO and LIN each at 0.5, 1.0 and 2.0% (v/v), MON at 2.5, 5.0 and 10.0 μg/ml; and YUCC, BENT and TANN each at 0.1, 0.2 and 0.4% (w/v), respectively.

[e] Within a row and parameter (L1–L3), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L3), marked values tend to differ from their respective control ($P < 0.10$).

[g] NP, not presented. Data not included due to interference of tannic acid with lactate assay (see Section 4).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table 3
Effects of oils and bioactive agents on fermentation characteristics of in vitro ruminal incubations (Exp. 2)

Parameter[c]	Level[d]	Control	Treatment[a]							S.E.	Level of significance[b]		
			PALM	SOY	SAL	T80	QUIL	FISH	LECI		T	L	T × L
pH	L1	6.88	6.80	6.77	6.86	6.87	6.79	6.86	6.82	0.072	**	*	***
	L2		6.95	6.77	6.90	6.86	6.98	6.95	6.80				
	L3		6.80	6.91	7.42[e]	6.83	6.97	6.86	6.74[f]				
Ammonia (mmol/l)	L1	26.7	27.1	27.3	23.2[e]	28.2[e]	27.2	32.1[e]	27.1	0.49	***	**	***
	L2		27.0	26.6	21.9[e]	27.7[f]	27.5	35.9[e]	26.3				
	L3		27.1	26.3	20.9[e]	28.5[e]	29.5[e]	39.8[e]	25.7[f]				
TFAA (mmol/l)	L1	7.0	6.7	6.6	10.9[e]	6.5	6.4	12.7[e]	7.6	0.99	***	***	***
	L2		7.5	6.7	13.7[e]	6.9	7.7	17.9[e]	6.9				
	L3		6.9	7.2	16.3[e]	7.2	7.8	27.7[e]	6.4				
RS (mmol/l)	L1	0.66	0.62	0.64	0.79[e]	0.62	0.68	0.80[e]	0.71	0.050	***	***	***
	L2		0.63	0.61	0.89[e]	0.65	0.91[e]	1.02[e]	0.64				
	L3		0.63	0.60	1.06[e]	0.65	1.17[e]	1.35[e]	0.70				
Soluble protein (g/l)	L1	4.4	4.7	4.8	5.2[e]	4.5	4.4	4.9[f]	4.8	0.23	***	*	*
	L2		4.6	4.7	4.8	4.6	4.9[f]	5.9[e]	5.0[f]				
	L3		4.6	4.8	4.6	4.7	5.5[e]	6.4[e]	5.2[e]				
VFA (mmol/l)													
Acetic		56.8	53.7	61.5	51.0	53.2	55.4	59.9	57.9	0.78	*	NS	NS
Propionic		25.2	24.1	27.6	24.5	23.4	24.1	25.8	24.5	0.35	NS	NS	NS
Isobutyric		1.46	1.40	1.55	1.25[e]	1.48	1.43	1.55	1.43	0.017	**	NS	NS
Butyric		10.3	10.1	10.7	8.7[e]	10.1	10.2	11.2	11.3[e]	0.14	***	NS	NS
iso-Valeric		2.93	2.86	3.04	2.42[e]	3.01	3.00	3.17	2.96	0.037	***	NS	NS
Valeric		3.06	3.01	3.10	1.91[e]	3.06	3.29	3.04	3.15	0.053	***	NS	NS
Total VFA		99.8	95.2	107.5	89.9	94.1	97.4	104.6	101.3	1.25	*	NS	NS
A:P		2.27	2.23	2.25	2.09[e]	2.28	2.30	2.32	2.37[e]	0.019	***	NS	NS

Lactate (mmol/l)	L1	0.13	0.21[e]	0.15	0.18[f]	0.12	0.20[e]	0.15	0.16	0.019	***	***	***
	L2		0.26[e]	0.18[f]	0.18[f]	0.13	0.39[e]	0.19[e]	0.15				
	L3		0.38[e]	0.21[e]	0.21[e]	0.16	0.62[e]	0.19[e]	0.13				

NS, not significant ($P > 0.05$).

[a] Abbreviations are defined in Table 1.

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] TFAA, total free amino acids; RS, reducing sugars; VFA, volatile fatty acids; A:P, acetate:propionate ratio.

[d] Levels L1–L3 refer to inclusion of PALM and SOY each at 0.5, 1.0 and 2.0% (v/v), SAL at 1.25, 2.5 and 5.0 μg/ml; T80 at 0.05, 0.1 and 0.2% (v/v), QUIL at 0.1, 0.2 and 0.4% (w/v), and FISH and LECI at 0.5, 1.0 and 2.0% (w/v), respectively.

[e] Within a row and parameter (L1–L3), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L3), marked values tend to differ from their respective control ($P < 0.10$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Compared with the respective controls, TFAA concentrations decreased ($P < 0.05$) with canola oil, bentonite and yucca and increased ($P < 0.05$) with fish meal and both ionophores. Concentrations of reducing sugars were increased ($P < 0.05$) by fish meal, salinomycin, and quillaja (except the lowest level). Soluble protein concentrations were reduced ($P < 0.05$) consistently by bentonite, but were increased ($P < 0.05$) by certain treatment levels of linseed oil and tannic acid (Exp. 1), and by fish meal, lecithin, salinomycin, and quillaja (Exp. 2).

As with ammonia, background VFA concentrations differed between the incubation groups, with Exp. 1 > Exp. 2 ($P < 0.05$; data not shown). In Exp. 1, bentonite and tannic acid reduced ($P < 0.05$) total VFA concentrations, relative to the control. Effects of bioactive agents on individual VFA concentrations (relative to the controls) were: tannic acid reduced ($P < 0.05$) acetate, propionate, acetate to propionate (A:P) ratio, isobutyrate, isovalerate, and valerate; bentonite reduced ($P < 0.05$) propionate, and isobutyrate (lowest and highest treatment levels only); monensin reduced ($P < 0.05$) isobutyrate, isovalerate (lowest and highest treatment levels only) and valerate; salinomycin reduced ($P < 0.05$) the A:P ratio and all of the C4 and C5 VFA; and lecithin increased ($P < 0.05$) butyrate and the A:P ratio. Significant ($P < 0.05$) treatment × level interactive effects on individual VFA concentrations were observed during Exp. 1 but not Exp. 2.

In all incubations, lactate concentrations were relatively low (except with tannic acid) but in general increased with treatment levels. Tannic acid yielded lactate concentrations of 1.95, 4.08 and 6.77 mmol/l at treatment levels L1–L3, respectively. With those data excluded from the statistical analysis (see Section 4), an effect of level ($P < 0.05$) was observed in Exp. 1, and in Exp. 2, highly significant effects ($P < 0.001$) of treatment, level, and treatment × level were observed. The overall effect of treatment was not significant ($P > 0.05$) in Exp. 1, but the highest level of canola oil and of linseed oil, and all levels of yucca, increased ($P < 0.05$) lactate concentrations compared to the control. In Exp. 2, lactate concentrations increased ($P < 0.05$) with all levels of quillaja, the higher levels of soybean oil and fish meal, and the highest level of salinomycin.

Compared to the respective controls, CMCase activity in the incubation medium was reduced ($P < 0.05$) in Exp. 1 by yucca, bentonite, and substantially (25%) by tannic acid (Table 4) and in Exp. 2 by all treatments except fish meal and Tween 80 (Table 5). Xylanase activity was linearly reduced ($P < 0.05$) by yucca ($R^2 = 0.99$), bentonite ($R^2 = 0.97$), and tannic acid ($R^2 = 0.96$). Xylanase was also reduced ($P < 0.05$) by canola oil, palm oil, salinomycin and quillaja, but was increased ($P < 0.05$) by fish meal and the two lower levels of coconut oil. The strongest inhibitory effect was exhibited by tannic acid, which reduced xylanase activity by 34–69%. All treatments, except coconut oil and fish meal, decreased ($P < 0.05$) amylase activity, whereas none of the treatments affected deaminative activity.

Protozoal populations were reduced ($P < 0.05$; $\log_{10}$ transformed data) only with the tannic acid, quillaja and lecithin treatments (Tables 6 and 7). Across treatments and incubations, protozoal populations comprised predominantly (84%) *Entodinium* spp. Incorporation of ^{15}N from casein into protozoal protein was dramatically reduced from 24 to 57% ($P < 0.05$) by monensin, fish meal, salinomycin, and the higher two levels of tannic acid, and to a lesser extent (8% reduction, $P < 0.05$) by the lowest level of lecithin. Protozoal incorporation of ^{15}N was increased ($P < 0.05$) by bentonite, quillaja, the higher two levels of yucca, and the highest dose of lecithin. All treatment levels of fish meal and salinomycin

Table 4
Effects of oils and bioactive agents on polysaccharide-degrading and deaminative activities of in vitro ruminal incubations (Exp. 1)

Parameter[c]	Level[d]	Control	Treatment[a]							S.E.	Level of significance[b]		
			CAN	COCO	LIN	MON	YUCC	BENT	TANN		T	L	T × L
CMCase[e]		39.6	37.0	41.2	38.5	37.6	35.1[f]	34.8[f]	29.7[f]	0.59	***	*	NS
Xylanase	L1	132.6	119.8[f]	152.0[f]	141.0	140.0	123.9	115.7[f]	87.8[f]	5.39	***	***	**
	L2		118.6[f]	149.0[f]	132.6	120.9[g]	119.3[f]	109.2[f]	78.2[f]				
	L3		119.7[f]	144.8[g]	127.1	128.6	112.4[f]	86.0[f]	41.0[f]				
Amylase		157.0	111.1[f]	163.4	125.3[f]	141.1[f]	141.5[f]	133.3[f]	85.8[f]	2.94	***	*	NS
Deaminative		4.54	4.98	4.82	5.29	4.77	4.76	4.48	4.23	0.114	NS	NS	NS

NS, not significant ($P > 0.05$).

[a] Abbreviations are defined in Table 1.

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] Polysaccharide-degrading activities are expressed as nmol of reducing sugars released from substrate by 1 ml of incubation medium in 1 min. Deaminative activity is expressed as μmol NH_3 released by 1 ml of incubation medium in 1 h.

[d] Levels L1–L3 refer to inclusion of CAN, COCO and LIN each at 0.5, 1.0 and 2.0% (v/v), MON at 2.5, 5.0 and 10.0 μg/ml; and YUCC, BENT and TANN each at 0.1, 0.2 and 0.4% (w/v), respectively.

[e] CMCase, carboxymethylcellulase.

[f] Within a row and parameter (L1–L3), marked values differ from their respective control ($P < 0.05$).

[g] Within a row and parameter (L1–L3), marked values tend to differ from their respective control ($P < 0.10$).

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table 5
Effects of oils and bioactive agents on polysaccharide-degrading and deaminative activities of in vitro ruminal incubations (Exp. 2)

Parameter[c]	Control	Treatment[a]							S.E.	Level of significance[b]		
		PALM	SOY	SAL	T80	QUIL	FISH	LECI		T	L	T × L
CMCase[d]	50.5	45.3[e]	46.8[e]	45.9[e]	51.3	43.1[e]	52.9	46.8[e]	0.63	***	NS	NS
Xylanase	161.6	146.6[e]	172.6	144.5[e]	163.5	144.3[e]	184.1[e]	150.2	2.23	***	NS	NS
Amylase	117.3	79.8[e]	93.1[e]	95.4[e]	97.4[e]	106.4[e]	112.3	80.6[e]	1.94	***	NS	NS
Deaminative	4.13	4.06	3.80	4.24	4.34	4.07	4.49	4.15	0.138	NS	NS	NS

NS, not significant ($P > 0.05$).

[a] Abbreviations of treatments are defined in Table 1. PALM and SOY were each included at 0.5, 1.0 and 2.0% (v/v), SAL at 1.25, 2.5 and 5.0 μg/ml; T80 at 0.05, 0.1 and 0.2% (v/v), QUIL at 0.1, 0.2 and 0.4% (w/v), and FISH and LECI at 0.5, 1.0 and 2.0% (w/v).

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] Polysaccharide-degrading activities are expressed as nmol of reducing sugars released from substrate by 1 ml of incubation medium in 1 min. Deaminative activity is expressed as μmol NH_3 released by 1 ml of incubation medium in 1 h.

[d] CMCase, carboxymethylcellulase.

[e] Within a row, marked values differ from their respective control ($P < 0.05$).

*** $P < 0.001$.

Table 6
Effects of oils and bioactive agents on protozoal numbers and ^{15}N incorporation into microbial protein during in vitro incubation (Exp. 1)

Parameter[c]	Level[d]	Control	Treatment[a]							S.E.	Level of significance[b]		
			CAN	COCO	LIN	MON	YUCC	BENT	TANN		T	L	T × L
Protozoal numbers		5.91 (8.4)	5.92 (8.4)	5.92 (8.3)	5.90 (8.0)	5.89 (7.9)	5.94 (8.9)	5.86 (7.4)	5.80[e] (6.7)	0.010	*	NS	NS
$^{15}N_{protozoa}$	L1	21.38	20.73	20.13[e]	21.33	13.35[e]	22.70[f]	24.60[e]	21.30	0.630	***	NS	***
	L2		20.77	21.05	21.23	16.18[e]	23.10[e]	25.83[e]	17.40[e]				
	L3		21.28	21.90	21.35	14.40[e]	23.00[e]	25.80[e]	14.85[e]				
$^{15}N_{bacteria}$	L1	32.08	31.90	31.55	32.28	29.98[e]	32.40	31.78	40.10[e]	0.896	***	NS	*
	L2		31.80	31.60	32.23	30.55	32.93	31.88	36.23[e]				
	L3		31.83	31.75	31.95	30.78	33.35	32.28	33.98[f]				
Prot:bact	L1	0.67	0.66	0.64[e]	0.67	0.45[e]	0.70[f]	0.78[e]	0.54[e]	0.015	***	NS	***
	L2		0.66	0.67	0.67	0.53[e]	0.70[f]	0.82[e]	0.48[e]				
	L3		0.68	0.69	0.68	0.47[e]	0.70[f]	0.81[e]	0.44[e]				

NS, not significant ($P > 0.05$).

[a] Abbreviations are defined in Table 1.

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] For protozoal numbers, means and S.E. were determined from $\log_{10}$ transformed data. Values in parentheses immediately to the right of the $\log_{10}$ means are the means calculated from actual data ($\times 10^5$ per ml). $^{15}N_{protozoa}$ and $^{15}N_{bacteria}$ are least square means of enrichments of ^{15}N (at.% excess) in protozoal and bacterial pellets, respectively. Prot:bact, proportion of protozoal N originating from bacterial N.

[d] Levels L1–L3 refer to inclusion of CAN, COCO and LIN each at 0.5, 1.0 and 2.0% (v/v), MON at 2.5, 5.0 and 10.0 μg/ml; and YUCC, BENT and TANN each at 0.1, 0.2 and 0.4% (w/v), respectively.

[e] Within a row and parameter (L1–L3), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L3), marked values tend to differ from their respective control ($P < 0.10$).

* $P < 0.05$.

*** $P < 0.001$.

Table 7
Effects of oils and bioactive agents on protozoal numbers and ^{15}N incorporation into microbial protein during in vitro incubation (Exp. 2)

Parameter[c]	Level[d]	Control	Treatment[a]							S.E.	Level of significance[b]		
			PALM	SOY	SAL	T80	QUIL	FISH	LECI		T	L	T × L
Protozoal numbers		6.05 (11.3)	5.96 (9.2)	6.02 (10.6)	5.92 (8.6)	5.99 (10.0)	5.61[e] (5.2)	5.96 (10.1)	5.90[e] (8.2)	0.021	***	NS	NS
$^{15}N_{protozoa}$	L1	16.38	16.20	16.50	10.75[e]	16.38	17.48[e]	10.73[e]	15.05[e]	0.432	***	NS	***
	L2		16.60	16.98	9.72[e]	16.52	18.58[e]	8.48[e]	16.98				
	L3		16.15	16.85	9.63[e]	16.25	19.58[e]	7.05[e]	18.95[e]				
$^{15}N_{bacteria}$	L1	27.21	26.80	27.18	25.78[e]	26.90	27.23	22.90[e]	26.30[e]	0.344	***	***	***
	L2		26.40[e]	27.05	24.78[e]	26.05[e]	26.23[e]	20.20[e]	26.83				
	L3		27.00	26.58	22.53[e]	24.60[e]	25.85[e]	17.98[e]	27.15				
Prot:bact	L1	0.61	0.61	0.62	0.42[e]	0.62	0.65[f]	0.47[e]	0.58	0.018	***	**	***
	L2		0.63	0.63	0.39[e]	0.64[f]	0.71[e]	0.42[e]	0.64				
	L3		0.60	0.64	0.43[e]	0.67[e]	0.77[e]	0.39[e]	0.70[e]				

NS, not significant ($P > 0.05$).

[a] Abbreviations are defined in Table 1.

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] For protozoal numbers, means and S.E. were determined from $\log_{10}$ transformed data. Values in parentheses immediately to the right of the $\log_{10}$ means are the means calculated from actual data ($\times 10^5$ per ml). $^{15}N_{protozoa}$ and $^{15}N_{bacteria}$ are least square means of enrichments of ^{15}N (at.% excess) in protozoal and bacterial pellets, respectively. Prot:bact, proportion of protozoal N originating from bacterial N.

[d] Levels L1–L3 refer to inclusion of PALM and SOY each at 0.5, 1.0 and 2.0% (v/v), SAL at 1.25, 2.5 and 5.0 μg/ml; T80 at 0.05, 0.1 and 0.2% (v/v), QUIL at 0.1, 0.2 and 0.4% (w/v), and FISH and LECI at 0.5, 1.0 and 2.0% (w/v), respectively.

[e] Within a row and parameter (L1–L3), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L3), marked values tend to differ from their respective control ($P < 0.10$).

** $P < 0.01$.

*** $P < 0.001$.

reduced ($P < 0.05$) incorporation of ^{15}N into bacterial protein, as did the two higher levels of quillaja and Tween 80. In contrast, tannic acid at the lower levels enhanced ($P < 0.05$) bacterial ^{15}N incorporation. The proportion of protozoal protein originating from bacterial protein was reduced ($P < 0.05$) with monensin and tannic acid and with salinomycin and fish meal. Conversely, the proportion of protozoal protein originating from bacterial protein was increased ($P < 0.05$) by bentonite, the higher two levels of quillaja and the highest levels of lecithin and Tween 80.

4. Discussion

4.1. Protozoal activity determination

Determinations of protozoal activity were based on the use of ^{15}N-casein as a N source to label ruminal bacteria and the presumption that ^{15}N tracer detected in the protozoal pellets was predominantly of bacterial origin. Ciliates have been reported in a number of studies (summarized by Williams and Coleman, 1992) to be unimportant in degradation of soluble protein in the rumen. *Isotricha* spp. can ingest both insoluble and soluble protein, but the entodiniomorphid protozoa digest only insoluble protein. A related study (Hristov et al., 2000a) revealed that *Entodinium* spp. accounted for 99.8% of the protozoa enumerated in inoculum prepared identically from the same donor heifers, thus it was anticipated that most (if not all) of the ^{15}N tracer appearing in the protozoa-rich fractions would have been of bacterial origin, i.e. from protozoal ingestion of bacterial protein, with only a small portion arising from direct incorporation of ^{15}N-casein by the protozoa. Good correlation ($P < 0.001$) between total protozoal numbers and the incorporation of ^{15}N into protozoal ($r = 0.83$) and bacterial ($r = 0.70$) fractions in that study attest to the validity of this approach (Hristov et al., 2000a; Hristov et al., unpublished data).

4.2. Feed oils

Dietary lipids are known to be potent modifiers of ruminal fermentation (Jenkins, 1993; Firkins, 1996), and their effects generally depend on the fatty acid composition of the lipid. Oils rich in saturated C12 and C14 and unsaturated C16 and C18 fatty acids inhibit ruminal protozoa in vitro and in vivo (Newbold and Chamberlain, 1988; Matsumoto et al., 1991; Machmüller and Kreuzer, 1999; Hristov et al., 2000a; Ivan et al., 2001).

The oils selected for the present study contained varying proportions of medium- and long-chain saturated and unsaturated fatty acids. Oleic (C18:1), linoleic (C18:2), linolenic (C18:3), lauric (C12:0) and palmitic (C16:0) acids account for approximately 60, 50, 47 45 and 40% of the fatty acids in canola, soybean, linseed, coconut and palm oils, respectively. However, under the conditions of this study, canola, linseed, palm and soybean oils exerted no effects on fermentation, other than reduced TFAA with canola oil and increased lactate with soybean and palm oils.

Dohme et al. (2000) reported that rumen ammonia, total VFA and acetate concentrations were unaffected by palm, coconut, and high lauric acid canola oils, that canola and coconut oils decreased propionate, and that coconut oil increased butyrate, as compared to a rumen

protected fat source. In a separate study, the same researchers observed that coconut oil reduced ruminal butyrate and valerate concentrations in lambs (Machmüller et al., 2000). In the present study, coconut oil at 0.5% (v/v) increased butyrate concentrations, but this effect was not evident at 1.0 or 2.0%. In a related study, Hristov et al. (2000a) found no effects of the sodium salts of oleic and palmitic acids on ruminal fermentation, but Na-laurate dramatically reduced ruminal ammonia and VFA concentrations, and increased the concentration of TFAA. In that study, effects of the unsaturates C18:2 and C18:3 were less pronounced.

Effects of oils on polysaccharide-degrading activities in the incubation media varied in the present study. Xylanase activity decreased with canola and palm oils, but increased with coconut oil, whereas all of the oils tested reduced amylase activity. In similar in vitro conditions, isolated unsaturated C18 fatty acids and lower levels (0.25 and 0.5%) of Na-laurate drastically increased amylase activity (Hristov et al., 2000a). Thus it appears that the inhibitory effect of the oils on amylase activity, observed in the present study, may have been due to factors other than their major fatty acids (e.g. minor fatty acids, other compounds, or physical properties of the oils).

None of the oils tested in this study affected protozoal counts or bacterial or protozoal incorporation of ^{15}N-casein. However, reports in the literature, as well as our own unpublished results, suggest strong antiprotozoal effects of feed oils and isolated fatty acids. Substantial reductions in ruminal protozoal counts have been reported in association with linseed, coconut, and canola oils (Newbold and Chamberlain, 1988; Machmüller and Kreuzer, 1999; Dohme et al., 2000; Machmüller et al., 2000), as well as lauric, linoleic, linolenic, and oleic acids (Newbold and Chamberlain, 1988; Matsumoto et al., 1991). Thus, it is possible that the observed effects of the oils on enzymatic activities may have led to decreased populations had the incubation been allowed to progress. In vitro ruminal protozoal populations were completely eradicated by lauric acid within 6 h and were sharply reduced by C18:1, C18:2 and C18:3 (Hristov et al., 2000a). More recently, we observed that high-linoleic acid safflower oil fed at 200 g/animal per day reduced ruminal protozoal populations to 42% of control levels in cattle fed high barley grain diets (Baah et al., 2002). The lack of effect of coconut oil, however, is somewhat surprising and may reflect insufficient duration or lipolytic activity in the incubation system to effect release of threshold levels of free fatty acids from this additive.

In the present closed fermentation study, incubations were terminated at 4 h to minimize deviance from in vivo conditions (e.g. as a result of end product accumulation, substrate depletion, etc.) on the basis of previous discussion (Hungate, 1966 (in reference to Markoff, 1913); Hristov and Broderick, 1994). This approach allowed direct comparison of a number of bioactive agents and assessment of treatment levels, but the short incubation period may have limited detection of treatment effects presenting later in the fermentation.

4.3. Surfactants

Lecithin (phosphatidylcholine) is a natural emulsifier and surfactant containing mostly linoleic acid together with smaller proportions of other palmitic, palmitoleic, oleic fatty acids. Fed at 5.2% of dietary DM to sheep, soybean lecithin reduced ruminal digestibilities of DM, ADF and dietary energy, and increased ruminal butyrate concentrations (Jenkins and

Fotouhi, 1990). In that study, ruminal protein digestion was severely depressed and ruminal ammonia concentrations decreased, but the efficiency of microbial protein synthesis was enhanced by the lecithin supplement. In the present study, however, no effects of lecithin on ruminal fermentation were apparent, other than a trend to increasing soluble protein levels with increasing lecithin concentration and, in agreement with Jenkins and Fotouhi (1990), an increased butyrate concentration. Lecithin was among the few bioactive agents tested that reduced protozoal populations (by 28%, relative to control). This effect most likely is due to the surfactant properties and fatty acid content of lecithin.

The surface-active agent investigated in this study, Tween 80 (polyoxyethylenesorbitan monooleate), has been reported to stimulate microbial fermentation and enzymatic activities (Helle et al., 1993) and to enhance the potency of ionphore antibiotics in pure culture (Hristov et al., 2000b). Increased fungal (Reese and Maguire, 1969) and bacterial (Lee et al., 1998; Kamande et al., 2000) enzyme activity in association with Tween 80 have been reported, but this was not apparent in the present study. Other than an increase in ammonia concentration, ruminal fermentation was unaffected by Tween 80, as were CMCase and xylanase activities, and amylase activity was actually depressed. An inhibitory effect on ^{15}N incorporation into bacterial protein was also demonstrated. It appears that the microbial response to Tween 80 in mixed ruminal culture may differ substantially from that observed in pure cultures.

4.4. Ionophores

The effects of ionophore antibiotics on ruminal fermentation have been well documented (Owens, 1980; Nagaraja et al., 1997) and include increased propionate production, and reduced methanogenesis, protein degradation, deamination of amino acids and ammonia concentration, as well as decreased lactic acid production and, therefore, reduced risk of acidosis (Nagaraja et al., 1997). In the present study, similar effects of monensin and salinomycin on protein metabolism were observed. All tested levels of both antibiotics resulted in decreased ammonia and increased TFAA concentrations, although soluble protein concentrations were unaffected. Total VFA, propionate and lactate accumulations were also not affected, but both ionophores inhibited accumulation of branched-chain VFA (BCFA). The BCFA in ruminal contents arise from degradation of branched-chain amino acids (Wallace, 1994) and so lower BCFA concentrations suggest inhibition of amino acid catabolism, but may also reflect enhanced utilization of BCFA by bacteria. In vivo, the rate of absorption of BCFA from the rumen would also influence their concentration in the ruminal contents.

Salinomycin apparently inhibited polysaccharide-degrading enzyme activities and bacterial incorporation of ^{15}N in the incubations, and both antibiotics substantially reduced the proportion of protozoal N arising from bacterial N, although neither ionophore affected total protozoal counts at 4 h. Others have reported suppression of ruminal ammonia and protozoal numbers by salinomycin (Kobayashi et al., 1990, 1992; McAllister et al., 1994), but those effects were not observed in a more recent in vivo study (Hristov et al., 2000b). It is possible, due to the short duration of the in vitro incubation, that only changes in protozoal activity rather than reduction in counts could be detected. Reduction in ammonia concentration and accumulation of free amino acids is typically occurring in the rumen of monensin (or salinomycin)-treated animals (Nagaraja et al., 1997). It is not clear why deaminative activity was not affected by the ionophores tested in the present study. The lack of

significant effect on total VFA concentration (numerically decreased by salinomycin) may indicate that only protein anabolic activities were inhibited by the ionophores. Under the conditions of the present study, salinomycin appeared to exert a stronger inhibitory effect on ruminal fermentation and on bacterial and protozoal activities than did monensin.

4.5. Saponin-containing plant products

Certain fermentation parameters were affected by the two plant products tested in this study. Yucca at 0.2 and 0.4% reduced ammonia concentrations by 15 and 27%, respectively, an effect often associated with saponin treatments in vitro and in vivo (Kil et al., 1994; Wallace et al., 1994; Hussain and Cheeke, 1995; Hristov et al., 1999b). Quillaja increased lactate concentrations, although in general they remained low in all treatments (<1 mM excepting tannic acid treatments, see below). Yucca and quillaja both reduced ruminal polysaccharide-degrading activities. This effect was not observed, however, when yucca powder was fed to heifers at ≤5.8 g/kg dietary DM (Hristov et al., 1999b).

Strong antiprotozoal effects have been reported previously for saponin-containing plants (Newbold et al., 1997). Yucca powder did not affect protozoal numbers in the present incubation, but reduced them by 42% in vivo (Hristov et al., 1999b). In contrast to the yucca treatments, quillaja reduced protozoal populations by 54% relative to the controls. Quillaja contains 10% saponins ((w/w), Desert King International, San Diego, CA), compared to 4.4% (w/w) saponins in yucca powder (Wang et al., 1998). Results of the present study suggest that *Q. saponaria* extract may be effective as a defaunating agent for cattle fed high grain diets. In another study, we reported that ruminal protozoal counts were reduced ($P < 0.01$) by 61% in cattle fed quillaja extract at 60 g/head per day, compared to controls (Baah et al., 2002). Work with saponin-containing plants, however, suggests that the effect on protozoa in the rumen may be transient rather than permanent, and inactivation by saliva may occur in vivo (Teferedegne, 2000).

4.6. Other bioactive agents

The most dramatic effects on ruminal fermentation and microbial activities were exerted by tannic acid (also known as gallotannin, which is a hydrolyzable tannin). All levels of inclusion (i.e. 0.1–0.4%) drastically reduced polysaccharide-degrading activities and concentrations of ammonia, as well as total and individual VFA, and increased concentrations of soluble proteins and reducing sugars. Tannins include the chemically distinct hydrolyzable tannins (HT) and condensed tannins (CT). The effects of CT on ruminal fermentation and microbial activities have been well documented (Barry and Leng, 1988; Bae et al., 1993; Chesson and Forsberg, 1997), but less information is available on HT. Tannic acid is toxic to ruminants, and high concentrations can cause ruminal stasis (Cheeke and Shull, 1985; Zhu and Flippich, 1995), although there is evidence that ruminal bacteria degrade tannic acid to yield gallic acid, pyrogallol and resorcinol as fermentation end products (Murdiati et al., 1992; Skene and Brooker, 1995; Singh et al., 2001). In the present study, tannic acid was one of the few additives that reduced protozoal counts. It also reduced protozoal incorporation of ^{15}N, but not that by bacteria. Reductions to protozoal populations in association with consumption of CT by ruminants, e.g. *Lotus corniculatus* grazed by sheep (Wang et al.,

1996) and *Quebracho* powder fed to cattle at 0.6% of dietary DM (Baah et al., 2002), have been attributed to the astringent nature of the CT, and possibly to inhibition of ruminal bacterial populations. Lowered polysaccharide-degrading activities in the present study may be indicative of inhibition of bacterial growth, but incorporation of N by bacteria was not impaired by tannic acid. Reduced enzymatic activity may have been due to the propensity of tannins to bind specifically to soluble proteins (Cheeke and Shull, 1985; Santos et al., 2000).

The dramatic increase in lactate concentrations in association with tannic acid most likely resulted from interference of the tannic acid with the lactate assay. The Goodall and Byers (1978) procedure is based on enzymatic hydrolysis of lactic acid to pyruvate, with a corresponding reduction of NAD to $NADH_2$. On the hypothesis that the strong protein-binding tendency of tannins in tannic acid may have affected the assay, a supplementary test was conducted that confirmed a strong interference of 0.1% tannic acid giving rise to readings corresponding to 5.9 mM lactate. Thus, tannic acid data were excluded from statistical analysis of the lactate concentrations (Table 2). Tannic acid also markedly increased concentrations of reducing sugars in the incubation media. A lesser interference of tannic acid with color reduction in the reducing sugars assay (ferricyanide assay, Hristov et al., 1998) was confirmed (0.1% tannic acid related to 1.03 mM reducing sugars), but analysis of the incubation samples suggested that tannic acid may have inhibited utilization of soluble sugars by ruminal miccroorganisms. Tannic acid was not found to interfere with any of the other assays in this study.

Increased concentrations of ammonia and soluble proteins observed in association with the fish meal treatment were probably a result of the substantial amount of protein introduced into the incubation media by the fish meal itself (approximately 14 mg/ml with 2% fish meal). Ammonia was not detected in diagnostic tests conducted with 0.5–2.0% (final concentrations) of an extract of fish meal. Further, TFAA concentrations ranging from 0.29 to 0.41 mM, with 0.5–2.0% fish meal extract, were negligible compared to those measured in fish meal treatments in Exp. 2 (Table 3). That the fish meal mediated increases in TFAA were far greater than the increases in ammonia concentrations indirectly suggests more intensive proteolysis and peptide hydrolysis than deamination, and possibly enhanced bacterial incorporation of ammonia from fish meal. Hoover et al. (1989) reported increased ammonia concentrations when continuous cultures were supplemented with fish meal at 6% of dietary DM, but they were unaffected when fish meal was supplemented at 9.7% of DM (Hussein et al., 1991). Including fish meal at 3% of dietary DM in alfalfa hay or silage diets did not influence ruminal ammonia or TFAA concentrations in dairy cows (Broderick, 1995). In the present study, fish meal increased xylanase activity and lactate concentrations, but it strongly inhibited bacterial and protozoal incorporation of ^{15}N. Hristov and Broderick (1994) attributed the reduced rate of ruminal protein degradation observed in vitro with fish meal to inhibition of microbial activities by fish oil. Hoover et al. (1989) also reported that fish oil reduced the efficiency of microbial protein synthesis, but this effect was not observed by Hussein et al. (1991). The depressed bacterial and protozoal incorporation of ^{15}N observed in the present study may support the above findings of microbial inhibition, but more likely reflects dilution of the ^{15}N-casein, amino acid/peptide and ammonia pools by unlabelled N from the fish meal. Fievez et al. (2001) reported inconsistent effects of fish oil on protozoal counts and microbial protein flow to the intestine (based on urinary excretion of purine derivatives) in sheep, and on methane production in complementary in vitro incubations.

Wallace and Newbold (1991) reported that bentonite strongly inhibited protozoal numbers, increased viable counts of bacteria, decreased concentrations of ammonia and VFA, and reduced degradation of *S. ruminantium* in the RUSITEC. These and other similar reported effects of bentonite have been attributed to adsorptive interactions between the bentonite and ruminal microorganisms or their growth substrates (Wallace and Newbold, 1991). A general pattern of inhibition of ruminal fermentation by bentonite (i.e. reduced TFAA, soluble protein and polysaccharide-degrading activities) was also observed in this study, but protozoal counts were not depressed. The enhanced incorporation of ^{15}N by protozoa and proportions of protozoal protein originating from bacterial protein observed seem to suggest stimulated protozoal uptake of bacterial N.

5. Conclusions

The oils tested in this study exerted negligible effects on ruminal fermentation in most cases, other than generally reducing polysaccharide-degrading activities in the incubation media, and did not affect protozoal numbers or ^{15}N incorporation into bacterial or protozoal cells. The ionophore antibiotics monensin and salinomycin apparently inhibited deamination and protozoal predation, and salinomycin also reduced acetate:propionate ratios, polysaccharide-degrading activities and bacterial incorporation of ^{15}N from casein, but neither antibiotic affected total protozoal counts. The effects of the two saponin-containing plants *Yucca schidigera* and *Quillaja saponaria* on ruminal fermentation were marginal, but they did markedly decrease polysaccharide-degrading activities. Both quillaja and lecithin decreased protozoal counts. Tannic acid exerted marked, and generally inhibitory, effects but did not affect bacterial incorporation of ^{15}N-casein. Applied at appropriate rates, some of the bioactive agents studied (e.g. quillaja extract, tannic acid, the ionophore antibiotics and, in particular, lecithin) may have the potential to reduce protozoal numbers, or protozoal predation of bacteria in the rumen, without negatively affecting ruminal fermentation or bacterial activities. Further study in continuous culture (>4 h) or in vivo could reveal whether the observed effects on enzymes and/or protozoal activities would result in decreased protozoal populations and possibly, improved feed efficiency.

Acknowledgements

This study was conducted with funding from the Canada/Alberta Beef Industry Development Fund. The authors thank the LRC barn staff for their conscientious care of the cattle and gratefully acknowledge the technical assistance of C. Barkley, W. Smart, C. Cockwill, and Z. Xu. This is Lethbridge Research Centre Contribution No. 38701055.

References

Abdullah, N., Hanita, H., Ho, Y.W., Kudo, H., Jalaludin, S., Ivan, M., 1995. The effects of bentonite on rumen protozoal population and rumen fluid characteristics of sheep fed palm kernel cake. Asian-Australas. J. Anim. Sci. 8, 249–254.

Baah, J., Hristov, A.N., McAllister, T.A., Ivan, M., Koenig, K.M., Rode, L.M., 2002. Evaluation of quillaja extract, quebracho tannin and safflower oil as selective defaunating agents in cattle. J. Anim. Sci. 80 (Suppl. 1), 404.

Bae, H.D., McAllister, T.A., Yanke, L.J., Cheng, K.-J., Muir, A.D., 1993. Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. Appl. Environ. Microbiol. 59, 2132–2138.

Barry, T.N., 1988. Condensed tannins: their role in the ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. In: Nolan, J.V., Leng, R.A., Demeyer, D.I. (Eds.), The Roles of Protozoa and Fungi in Ruminant Digestion. Penambul Books, Armidale, NSW, pp. 153–169.

Broderick, G.A., 1995. Performance of lactating dairy cows fed either alfalfa silage or alfalfa hay as the sole forage. J. Dairy Sci. 78, 320–329.

Burggraaf, W., Leng, R.A., 1980. Antiprotozoal effects of surfactant detergents in the rumen of sheep. N. Z. J. Agric. Res. 23, 287–291.

CCAC, 1993. Guide to the care and use of experimental animals. In: Olfert, E.D., Cross, B.M., McWilliam, A.A. (Eds.), Canadian Council on Animal Care, vol. 1, second ed., Ottawa, Ont., Canada.

Cheeke, P.R., Shull, L.R., 1985. Natural Toxicants in Feed and Poisonous Plants. AVI Publishing Company Inc., Westport, CT.

Chesson, A., Forsberg, C.W., 1997. Metabolism of nitrogen-containing compounds. In: Hobson, P.N., Stewart, C.S. (Eds.), The Rumen Microbial Ecosystem. Blackie Academic and Professional, London, UK, pp. 329–381.

Dohme, F., Machmüller, A., Wasserfallen, A., Kreuzer, M., 2000. Comparative efficiency of various fats rich in medium-chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. Can. J. Anim. Sci. 80, 473–482.

Fievez, V., Danneels, M., Demeyer, D., 2001. Effect of rumen fish oil injection on in vivo digestion and fermentation characteristics: differences in animal response. Proc. Nutr. Soc. 60, 75A (Abstract).

Firkins, J.L., 1996. Maximizing microbial protein synthesis in the rumen. J. Nutr. 126, 1347S–1354S.

Goodall, S.R., Byers, F.M., 1978. Automated micro method for enzymatic L(+) and D(−) lactic acid determination in biological fluids containing cellular extracts. Anal. Biochem. 89, 80–86.

Helle, S.S., Duff, S.J.B., Cooper, D.G., 1993. Effect of surfactants on cellulose hydrolysis. Biotechnol. Bioeng. 42, 611–617.

Hoover, W.H., Miller, T.K., Stokes, S.R., 1989. Effects of fish meals on rumen bacterial fermentation in continuous culture. J. Dairy Sci. 72, 2991–2998.

Hristov, A.N., Broderick, G.A., 1994. In vitro determination of ruminal protein degradability using [15]N-ammonia to correct for microbial nitrogen uptake. J. Anim. Sci. 72, 1344–1354.

Hristov, A.N., Broderick, G.A., 1996. Synthesis of microbial protein in ruminally cannulated cows fed alfalfa silage, alfalfa hay, or corn silage. J. Dairy Sci. 79, 1627–1637.

Hristov, A.N., McAllister, T.A., Cheng, K.-J., 1998. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzymes on rumen fermentation and nutrient digestibility. J. Anim. Sci. 76, 3146–3156.

Hristov, A.N., McAllister, T.A., Cheng, K.-J., 1999a. Effect of diet, digesta processing, freezing and extraction procedure on some polysaccharide-degrading activities of ruminal contents. Can. J. Anim. Sci. 79, 73–81.

Hristov, A.N., McAllister, T.A., Van Herk, F.H., Cheng, K.-J., Newbold, C.J., Cheeke, P.R., 1999b. Effect of *Yucca schidigera* extract on rumen fermentation and nutrient digestion in heifers. J. Anim. Sci. 77, 2554–2563.

Hristov, A.N., Ivan, M., McAllister, T.A., 2000a. In vitro effects of common fatty acids on protozoal numbers and activity and fermentation in ruminal fluid from cattle fed a barley-based diet. J. Anim. Sci. 78 (Suppl. 1), 275.

Hristov, A.N., McAllister, T.A., Olson, M.E., Yanke, L.J., Shelford, J.A., 2000b. Effect of Tween 80 and salinomycin on ruminal fermentation and nutrient digestion in steers fed a diet containing 70% barley. Can. J. Anim. Sci. 80, 363–372.

Hristov, A.N., Ivan, M., Rode, L.M., McAllister, T.A., 2001. Fermentation characteristics and ruminal ciliate protozoal populations in cattle fed medium or high concentrate barley-based diets. J. Anim. Sci. 79, 515–524.

Hristov, A.N., McAllister, T.A., Xu, Z., Newbold, C.J., 2002. Proteolytic activity in ruminal fluid from cattle fed two levels of barley grain: a comparison of three methods of determination. J. Sci. Food Agric. 82, 1886–1893.

Hungate, R.E. 1966. The Rumen and its Microbes. Academic Press, New York, p. 258.

Hussain, I., Cheeke, P.R., 1995. Effect of dietary *Yucca schidigera* extract on rumen and blood profiles of steers fed concentrate-or roughage-based diets. Anim. Feed. Sci. Technol. 51, 231–242.

Hussein, H.S., Stern, M.D., Jordan, R.M., 1991. Influsence of dietary protein and carbohydrate sources on nitrogen metabolism and carbohydrate fermentation by ruminal microbes in continuous culture. J. Anim. Sci. 69, 2123–2133.

Ivan, M., Daynellde, M.S., Mahadevan, S., Hidiroglou, M., 1992. Effect of bentonite on wool growth and nitrogen metabolism in fauna-free and faunated sheep. J. Anim. Sci. 70, 3194–3202.

Ivan, M., Mir, P.S., Koenig, K.M., Rode, L.M., Neill, L., Entz, T., Mir, Z., 2001. Effects of dietary sunflower seed oil on rumen protozoa population and tissue concentration of conjugated linoleic acid in sheep. Small Rumin. Res. 41, 215–227.

Jenkins, T.C., 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76, 3851–3863.

Jenkins, T.C., Fotouhi, N., 1990. Effects of lecithin and corn oil on site of digestion, ruminal fermentation and microbial protein synthesis in sheep. J. Anim. Sci. 68, 460–466.

Jouany, J.-P., 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J. Nutr. 126, 1335S–1346S.

Kamande, G., Baah, J., Shelford, J.A., Cheng, K.-J., McAllister, T.A., 2000. Effects of Tween 60 and Tween 80 on protease activity, thiol group reactivity, protein adsorption, and cellulose degradation by rumen microbial enzymes. J. Dairy Sci. 83, 536–542.

Kil, J.Y., Cho, N.K., Kim, B.S., Lee, S.R., Maeng, W.J., 1994. Effects of Yucca extract addition on the in vitro fermentation characteristics of feed and feces, and on the milk yields in lactating cows. Kor. J. Anim. Sci. 36, 698–709.

Kobayashi, Y., Wakita, M., Sakauchi, R., Hoshino, S., 1990. Effects of ionophores on rumen microbes and host animal nutrition. In: Hoshino, S., Onodera, R., Minato, H., Itabashi, H. (Eds.), The Rumen Ecosystem: the Microbial Metabolism and its Regulation. Japan Sci. Soc. Press, Tokyo, pp. 179–186.

Kobayashi, Y., Wakita, M., Hoshino, S., 1992. Effects of ionophore salinomycin on nitrogen and long-chain fatty acid profiles of digesta in the rumen and the duodenum of sheep. Anim. Feed Sci. Technol. 36, 67–76.

Lee, S.S., Kim, S.W., Kim, H.D., Ha, J.K., Goto, M., Cheng, K.-J., 1998. Novel potential use of surfactant Tween 80 as a feed additive for ruminant animals. In: Proceedings of the 8th World Conference on Animal Production, Seoul, Korea, pp. 356–357.

Machmüller, A., Kreuzer, M., 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65–72.

Machmüller, A., Ossowski, D.A., Kreuzer, M., 2000. Comparative evaluation of the effects of coconut oil, oilseed and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41–60.

Markoff, I., 1913. Biochem. Z. 57, 1–70 (as cited by Hungate, R.E. 1966. The Rumen and its Microbes. Academic Press, New York, New York, p. 258).

Matsumoto, M., Kobayashi, T., Takenaka, A., Itabashi, H., 1991. Defaunation effects of medium-chain fatty acids and their derivatives on goat rumen protozoa. J. Gen. Appl. Microbiol. 37, 439–445.

McAllister, T.A., Moustafa, S.M.S., Cheng, K.-J., Newbold, C.J., McKain, N., Wallace, R.J., 1994. Effect of salinomycin on fermentation and nitrogen metabolism in the artificial rumen. Can. J. Anim. Sci. 74, 575–578.

McDougall, E.I., 1948. The composition and output of sheep's saliva. Biochem. J. 43, 99–109.

Muhammed, S., Stewart, C.S., Acamovic, T., 1994. Effects of tannic acid on cellulose degradation, adhesion and enzymic activity of rumen microorganisms. In: Proceedings of the International Symposium on Ruminant Physiology, Society of Nutritional Physiology, Frankfurt, Germany, p. 174.

Murdiati, T.B., McSweeney, C.S., Lowry, J.B., 1992. Metabolism in sheep of gallic acid, tannic acid and hydrolysable tannin from *Terminalia oblongata*. Aust. J. Agric. Res. 43, 1307–1319.

Nagaraja, T.G., Newbold, C.J., Van Nevel, C.J., Demeyer, D.I., 1997. Manipulation of ruminal fermentation. In: Hobson, P.N., Stewart, C.S. (Eds.), The Rumen Microbial Ecosystem. Blackie, London, UK, pp. 523–623.

Newbold, C.J., Chamberlain, D.G., 1988. Lipids as rumen-defaunating agents. Proc. Nutr. Soc. 43, 154A.

Newbold, C.J., El Hassan, S.M., Wang, J., Ortega, M.E., Wallace, R.J., 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Br. J. Nutr. 78, 237–249.

Ogimoto, K., Imai, S., 1981. Atlas of Rumen Microbiology. Japan Sci. Soc. Press, Tokyo, p. 158.

Owens, F.N., 1980. Ionophore effect on utilization and metabolism of nutrients–ruminants. In: Proceedings of the Georgia Nutritional Conference, University of Georgia, Athens, p. 17.

Reese, E.T., Maguire, A., 1969. Surfactants as stimulants of enzyme production by microorganisms. Appl. Microbiol. 17, 242–245.

Santos, G.T., Oliveira, R.L., Petit, H.V., Cecato, U., Zeoula, L.M., Rogolon, L.P., Damasceno, J.C., Branco, A.F., Bett, V., 2000. Effect of tannic acid on composition and ruminal degradability of bermudagrass and alfalfa silages. J. Dairy Sci. 83, 2016–2020.

SAS, 1996. SAS/STAT® Software: Changes and Enhancements through Release 6.11. SAS Institute Inc., Cary, NC, 1104 pp.

Singh, B., Bhat, T.K., Sharma, O.P., 2001. Biodegradation of tannic acid in an in vitro ruminal system. Livest. Prod. Sci. 68, 259–262.

Skene, I.K., Brooker, J.D., 1995. Characterization of tannin acylhydrolase activity in the ruminal bacterium *Selenomonas ruminantium*. Anaerobe 1, 321–327.

Teferedegne, B., 2000. New perspectives on the use of tropical plants to improve ruminant nutrition. Proc. Nutr. Soc. 59, 209–214.

Wallace, R.J., 1994. Amino acid and protein synthesis, turnover, and breakdown by ruminal microorganisms. In: Asplund, J.M. (Ed.), Principles of Protein Nutrition of Ruminants. CRC Press, Boca Raton, FL, pp. 71–113.

Wallace, R.J., Newbold, C.J., 1991. Effects of bentonite on fermentation in the rumen simulation technique (Rusitec) and on ciliate protozoa. J. Agric. Sci. (Camb.) 116, 163–168.

Wallace, R.J., Arthaud, L., Newbold, C.J., 1994. Influence of *Yucca shidigera* [sic] extract on ruminal ammonia concentrations and ruminal microorganisms. Appl. Environ. Microbiol. 60, 1762–1767.

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry, T.N., Foote, A.N., Purchas, R.W., 1996. Effects of condensed tannins upon the performance of lambs grazing *Lotus corniculatus* and lucerne (*Medicago sativa*). J. Agric. Sci. (Camb.) 126, 87–98.

Wang, Y., McAllister, T.A., Newbold, C.J., Rode, L.M., Cheeke, P.R., Cheng, K.J., 1998. Effects of *Yucca schidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). Anim. Feed Sci. Technol. 74, 143–153.

Williams, A.G., Coleman, G.S., 1992. The Rumen Protozoa. Springer, New York, 441 pp.

Zhu, J., Flippich, L.J., 1995. Acute intra-abomasal toxicity of tannic acid in sheep. Vet. Human Toxicol. 37, 50–54.