

## Effect of dietary *Enterolobium cyclocarpum* on microbial protein flow and nutrient digestibility in sheep maintained fauna-free, with total mixed fauna or with *Entodinium caudatum* monofauna

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Three groups of five wethers with ruminal and duodenal cannulas and maintained as either fauna-free (FF) or inoculated with total mixed fauna (TF) or *Entodinium caudatum* as a single-species monofauna (EN) were used in an experiment with two 28 d periods. In the first period, the sheep were fed a control barley-based diet (40:60 concentrate to silage DM) and in the second period the diet was supplemented with 187 g DM of *Enterolobium cyclocarpum* for the last 12 d of the period. The diets of period 1 and 2 were isonitrogenous. There was no effect of fauna on apparent ruminal and total tract organic matter and fibre digestion, but bacterial and microbial N flow and efficiency were improved in FF sheep compared to TF sheep. In period 2, protozoal numbers were reduced between 31 and 88% 2 h after feeding *E. cyclocarpum* for the third to twelfth day of supplementation and by an average of 25% in samples collected over the 24 h feeding cycle. Supplementation of the diet with *E. cyclocarpum* and the consequent protozoal reduction in TF and EN sheep improved the flow of non-ammonia N and bacterial N to the small intestine and the efficiency of microbial synthesis. However, *E. cyclocarpum* reduced ruminal organic matter digestion, especially in faunated sheep, and total tract organic matter, N and fibre digestion. Thus, a reduction in the protozoal cell numbers of 25% was sufficient to achieve the beneficial effects of reduced fauna on the bacterial protein supply, but diet digestibility was reduced.

***Enterolobium cyclocarpum*: Reduced fauna: Ruminal ciliate protozoa: Microbial protein: Sheep**

Secondary metabolites are found in many plants and confer a mechanism of chemical defence against microorganisms, insects and grazing ruminants. Initial interest in feeding secondary plant metabolites to manipulate digestive function in ruminants began with identifying forages that could serve as cost-effective supplements for ruminants on poor-quality pastures or crop residues<sup>1</sup>. More recently, interest in the antimicrobial properties of secondary plant metabolites has diversified as the livestock industry strives to find natural alternatives to the use of growth-promoting antibiotics to maintain animal health and environmental sustainability<sup>2</sup>.

Saponins are one type of secondary metabolites and occur as either triterpenoid or steroid aglycone structures linked to various carbohydrate moieties. The chemical structures of

the saponins in *Enterolobium cyclocarpum*, a tropical multi-purpose tree, are unknown<sup>3</sup>, but other saponin-containing plants of the Leguminosae family generally have saponins of a triterpenoid-type structure<sup>4</sup>. One of the mechanisms of action of saponins that may be of beneficial importance in ruminant nutrition is the antiprotozoal activity. The detrimental effects of saponins on ciliate protozoa are thought to be due to binding of the saponin with sterols present on the protozoal membrane surface causing cell rupture<sup>3</sup>. Removal of ruminal ciliate protozoa either completely, partially or selectively by specific genera offers a potential approach to increase N availability and efficiency of utilization, and to reduce N excretion in manure of ruminant livestock. Microbial protein is the major source of amino acids entering the small intestine and

**Abbreviations:** ADF, acid detergent fibre; EN, *Entodinium caudatum* monofauna; FF, fauna-free; NAN, non-ammonia N; NDF, neutral detergent fibre; NMNAN, non-microbial non-ammonia N; OM, organic matter; TF, total mixed fauna; VFA, volatile fatty acids.

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accounts for between 35 and 90% of the protein flow<sup>5</sup>. The amount of microbial protein reaching the intestine, however, may represent only one-half of the total amount of microbial protein that is synthesized within the rumen<sup>6,7</sup>. The effect of protozoal predation on ruminal bacterial numbers and microbial protein flow is considerable. It has been shown that the removal of protozoa can result in increased bacterial numbers in the rumen, reduced intraruminal microbial protein recycling, and a 20–60% increase in the flow of bacterial protein from the rumen<sup>7,8</sup>.

Ruminal ciliate protozoa are categorized as entodiniomorphs and holotrichs based on their physical properties and metabolism<sup>9</sup>. The entodiniomorphs form the majority of the protozoal community in the rumen and feed principally by engulfment of particulate matter of which bacteria serve as the major protein source<sup>9</sup>. Holotrich protozoa (e.g. *Isotricha* spp. and *Dasytricha* spp.) utilize soluble protein sources but may also ingest and digest bacteria<sup>9</sup>. Protozoa of the genus *Entodinium* are the most numerous comprising between 78 and 98% of the protozoa in a mixed protozoal community<sup>10–13</sup>. The *Entodinia* have been found to be the major contributor to bacterial protein turnover in the rumen<sup>14,15</sup>.

Under experimental conditions the complete absence of ruminal ciliate protozoa improves the protein nutrition of the ruminant<sup>7,8</sup>. Under practical conditions, however, it is unlikely that complete protozoal removal can be achieved or maintained. Feeding the foliage of *E. cyclocarpum* causes a reduction in ruminal ciliate protozoal numbers in sheep<sup>1,16</sup>, although the antiprotozoal effect is of short duration, lasting only 12–14 d, and most effective in animals that have not been previously exposed to the antiprotozoal agent<sup>12</sup>. Due to the short duration of the antiprotozoal effect of *E. cyclocarpum*, it would have limited value as an antiprotozoal agent in the field, but it may be useful for short-term studies to determine the effect of a partial reduction of protozoal cell numbers on the protein nutrition of the ruminant. The objectives of the present study were to determine if a reduction in ruminal fauna due to *E. cyclocarpum* feeding can improve bacterial and microbial protein flow to the intestine and nutrient digestion in sheep faunated with total mixed fauna (TF) or *Entodinium caudatum* monofauna (EN). The effects were measured during the period when *E. cyclocarpum* was known to be effective at reducing protozoal numbers in the rumen of sheep<sup>12</sup>.

## Materials and methods

### Animals and housing

Fifteen 1.5-year-old Canadian Arcott fauna-free (FF) wethers fitted with ruminal and duodenal (placed proximal to the common bile and pancreatic ducts) cannulas were used. The ruminal cannulas measured 26 mm in internal diameter and were constructed of natural rubber (Macam Rubber Pty. Ltd, Baulkham Hills, NSW, Australia). The duodenal cannulas were a simple T-type with an 11 mm internal diameter, gutter-type flanges and constructed of polyvinylchloride.

At birth, the sheep were removed from the dam and reared as a group in a facility separated from other ruminants to maintain their natural FF status. The sheep were used in another study<sup>17</sup>, prior to their use in the present experiment.

For the prior study<sup>17</sup>, the fifteen cannulated FF sheep were inoculated with 50 ml each of ruminal contents collected and combined from three donor sheep with a natural bacterial community. The bacterial community in the donor sheep was established by inoculation with ruminal contents that were collected from a naturally faunated cow and frozen to kill the protozoa but maintain the majority of the bacteria. Ten days after bacterial inoculation, the sheep were then randomly assigned to three groups of five sheep each and maintained as either FF or inoculated with EN or *Polyplastron multivesiculatum* genera to determine the effects of the monofaunas on the duodenal flow of bacterial and protozoal N, and their contribution to ruminal fermentation and digestion of feed<sup>17</sup>. At the termination of the prior study, the FF sheep and the sheep inoculated with EN were maintained with their respective fauna communities for use in the present experiment. The sheep that were monofaunated with *Polyplastron* were then inoculated with 50 ml of the ruminal fluid containing TF on the first day of the first period of the present experiment. The ruminal fluid used for the inoculation originated from three donor sheep faunated with mixed fauna of the Type A population<sup>18</sup>.

For the duration of the experiment, the sheep were housed in an enclosed barn separated from other ruminants. The sheep were cared for according to the guidelines of the Canadian Council on Animal Care<sup>19</sup> and the experimental protocol was approved by the Lethbridge Centre Animal Care Committee.

The experiment consisted of two periods of 28 d each with measurements and sample collection performed during the last 16 d (Fig. 1). The sheep were housed in individual pens in separate rooms of the barn for each protozoal group for the first 19 d of each period. The sheep were then housed in metabolism crates for four consecutive days: 1 d for adjustment to the crates and 3 d for total faecal collection. At the end of the 4 d, the sheep were returned to their individual pens for a minimum of 4 h in accordance with the guidelines of the Canadian Council on Animal Care, and then placed back into the metabolism crates to complete the remaining 5 d of the period. The metabolism crates were separated by a minimum distance of 2 m between the protozoal treatment groups.

Body weight was measured at the beginning and end of the first period, and again at the end of the second period at the same time on each day and averaged 68.2 (SD 6.0) kg over the experiment.

### Dietary treatments

The dietary treatments were offered twice daily with one-third of the feed offered at 08.30 hours and two-thirds offered at 15.30 hours. For days 1–15, feed was offered in an amount to permit an *ad libitum* level of intake by ensuring that approximately 10% of the feed offered was refused each day. From day 16 to day 28, the diet was offered at 85% of the mean daily intake of the sheep with the lowest amount of intake as determined from days 1–15. Sheep had free access to water at all times.

The basal diet consisted of (DM basis) barley silage (600 g/kg) and a barley grain and soyabean meal pelleted concentrate (400 g/kg) with sufficient minerals and vitamins to meet

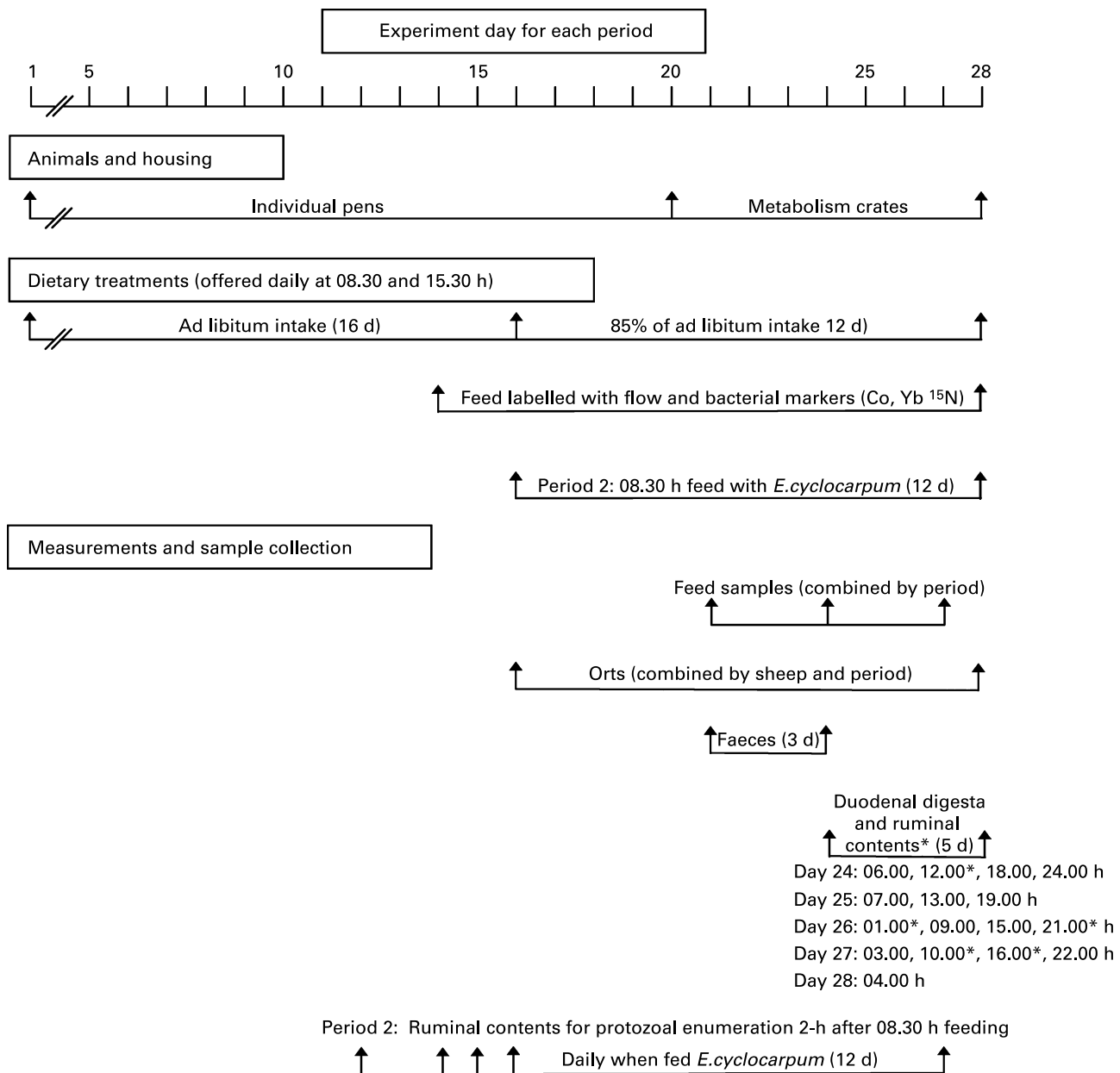


Fig. 1. Time line for the two 28 d periods of the experiment.

recommendations<sup>20</sup> (Table 1). The silage and concentrate were mixed daily prior to the morning feeding in a Data Ranger (American Calan, Northwood, NH, USA). In the first period, an additional 42.5 g soyabean meal (DM basis) was mixed into the 08.30 hour feed allotment for 12 d beginning on day 16 and continuing until the end of the period to make the diet (control) isonitrogenous to the diet fed in the second period. In the second period, 187 g (DM basis) of the basal diet was replaced with an equivalent amount of *E. cyclo carpum* leaves (187 g DM basis, 200 g as-fed basis) and was offered with the morning feed allotment for 12 d beginning on day 16 and continuing until the end of the period. Sun-dried branches of the *E. cyclo carpum* were imported from Costa Rica. The dried leaves were separated from the branches and twigs by hand. The saponin content of the *E. cyclo carpum*

was 0.80 mg/g DM as determined by the haemolysis of sheep blood with digitonin as a standard<sup>21</sup>. The sheep used in the present study had no prior exposure to *E. cyclo carpum*.

The flow of digesta to the duodenum was determined using Yb (particle-associated marker) and Co (solute marker) as dual markers<sup>22,23</sup>. <sup>15</sup>N and phosphatidylcholine were used as bacterial and protozoal markers for the calculation of the duodenal flow of bacterial and protozoal N, respectively. The flow and bacterial markers were incorporated into the concentrate prior to pelleting. About 1.3 kg YbCl<sub>3</sub>.6H<sub>2</sub>O (Rhône Poulenc Basic Chemicals Co., Shelton, CT, USA), 2.05 kg of the lithium salt of CoEDTA<sup>24</sup> and 300 g [<sup>15</sup>NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub> (minimum 10 atom percentage <sup>15</sup>N; Isotec Inc., Miamisburg, OH, USA) were dissolved in 10 litres water and sprayed on 350 kg concentrate.

**Table 1.** Ingredient and chemical composition of the basal diet, and the chemical composition of the *Enterolobium cyclocarpum* and soyabean meal

	Basal diet	<i>E. cyclocarpum</i>	Soyabean meal
Ingredient composition* (g/kg DM)			
Barley silage	600.0		
Barley grain (coarsely ground)	343.2		
Soyabean meal	30.1		
Dicalcium phosphate	4.1		
Calcium carbonate	7.0		
Trace mineralized salt†	6.1		
Vitamins A, D and E‡	0.2		
Molasses	4.5		
Zeolite§	4.5		
Flavour¶	0.2		
Chemical composition (g/kg DM)			
DM	490	932	943
OM	933	918	927
CP	122	231	500
NDF	333	589	97
ADF	184	486	33

ADF, acid detergent fibre; CP, crude protein; NDF, neutral detergent fibre; OM, organic matter.

\* All ingredients, with the exception of the barley silage, were pelleted (6.4 mm in diameter).

† Contained per kg of trace mineralized salt: 923 g NaCl, 50 g/kg Dynamate (Pitman Moore, Inc., Mendelein, IL, USA; 220 g/kg S, 180 g/kg K, 110 g/kg Mg, 10 g/kg Fe), 3.3 g Zn, 2.5 g Mn, 0.34 g Cu, 0.11 g I, 93 mg Co, 14.3 mg Se.

‡ Contained per g of vitamin mix: 3.44 mg retinyl acetate, 25 µg cholecalciferol, 100 mg α-tocopherol acetate.

§ Bear River Zeolite of Canada Corporation, Lethbridge, Alberta, Canada.

¶ ACF Flavouring Agent, Alltech, Inc., Guelph, Ontario, Canada.

From days 14 to 28 of the period, the sheep received the marked concentrate in place of unmarked concentrate. On day 14, prior to offering the marked diet, each sheep received a prime dose containing half of the amount of marker consumed per day. The prime dose of the markers (1.4 g of the lithium salt of CoEDTA, 0.87 g YbCl<sub>3</sub>·6H<sub>2</sub>O, 0.25 g [<sup>15</sup>NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>) was dissolved in 100 ml deionized water and administered to the rumen through the cannula.

#### Measurements and sample collection

Precautions were taken to avoid cross-contamination of the ruminal protozoal fauna between the treatments groups during feeding, sample collection and processing. Coveralls and gloves were changed between the handling and sample collection for each protozoal treatment group. The FF group was fed and sampled first, followed by the EN group and then the TF group of sheep. One room was set up for each protozoal treatment group with all the supplies and equipment necessary for sample processing.

#### Feed and orts samples

Samples of the barley silage and marked concentrate were collected on days 21, 24 and 27, dried in a 55°C oven for 48 h to determine DM content, ground through a 1 mm sieve and combined by period. Any feed refused when sheep were on a restricted intake from days 16 to 28 was weighed, sub-sampled, dried and ground as described for the feed samples

and combined by sheep and period. Samples of the soyabean meal and *E. cyclocarpum* were also collected, dried and ground. Feed and orts were analysed for DM, organic matter (OM), N, neutral detergent fibre (NDF), acid detergent fibre (ADF), and the digesta flow and bacterial markers.

#### Faeces and duodenal digesta collection

On day 20 the sheep were moved into metabolism crates and fitted with harnesses and faecal collection bags. The sheep were previously exposed to the crates and faecal collection bags<sup>17</sup>, and were allowed 1 d to become accustomed to the crates and bags prior to sample collection in the present experiment. Total collection of faeces was performed for three consecutive 24 h periods from days 21 to 24 (the fifth to eighth day of feeding *E. cyclocarpum*) to determine total tract nutrient digestibility based on the flow of the Yb marker<sup>23</sup>. The faecal output was collected daily and weighed; 10% was dried at 55°C in an oven to determine DM content, ground through a 1 mm sieve, and combined by sheep and period for analysis (DM, OM, N, NDF, ADF and Yb).

Duodenal samples were collected at various times from days 24 to 28 of each period (the eighth to the twelfth day of feeding *E. cyclocarpum*) to obtain digesta representative of the 24 h feeding cycle. Duodenal digesta was collected every 6 h, moving ahead 1 or 2 h each day to collect samples at 16 different times. Duodenal digesta (100 ml) was collected at 06.00, 12.00, 18.00 and 24.00 hours on day 24, at 07.00, 13.00 and 19.00 hours on day 25, at 01.00, 09.00, 15.00 and 21.00 hours on day 26, at 03.00, 10.00, 16.00 and 22.00 hours on day 27, and at 04.00 hours on day 28. Duodenal samples (100 ml for each time-point) were combined by sheep and period as collected and immediately frozen.

The combined duodenal samples were later thawed and a volume of 700–800 ml was separated into solid and liquid fractions by centrifugation at 1000 g, 4°C, for 15 min. The DM content of the fractions was determined by freeze-drying. The fractions were then ground through a 1 mm sieve. Marker and nutrient concentrations were determined in the whole, solid and liquid fractions of duodenal digesta to determine nutrient flow to the duodenum and pre-intestinal digestibility. An additional sample of the duodenal contents (15 ml) was centrifuged at 20 000 g and 4°C for 15 min and the supernatant was stored frozen until analysis of ammonia N.

#### Ruminal fermentation characteristics and bacterial and protozoal isolation

Ruminal contents for the determination of fermentation characteristics, and protozoal and bacterial isolation were collected at six different time-points (07.00, 10.00, 12.00, 16.00, 21.00 and 01.00 hours) from days 24 to 28. Ruminal contents (500 ml) were obtained through the cannula from multiple sites using a 60 cm<sup>3</sup> syringe attached to tubing (20 mm internal diameter). The pH of the ruminal contents collected was measured immediately using a pH metre. Ruminal contents (60–70 ml) were then strained through one layer of cheese-cloth. A volume of 5 ml of the strained ruminal fluid was acidified with 1 ml chilled 25% (w/v) meta-phosphoric acid and the samples were stored frozen (–30°C) until analysis of volatile fatty acids (VFA). A volume of 10 ml of the ruminal fluid



was added to 1 ml chilled 65% (w/v) TCA to acidify the sample and precipitate the protein. The samples were kept in an ice-bath for 30 min, centrifuged at high speed (20 000 g, 4°C, for 20 min), and the supernatant was stored frozen until analysis of ammonia N. A volume of 5 ml of the strained ruminal fluid was mixed with 5 ml methylgreen formalin saline<sup>25</sup> and the preserved samples were stored at room temperature in darkness until total cell numbers and species of protozoa were counted. In addition, during period 2, ruminal fluid samples were collected 2 h after the morning feed beginning 4 d before (days 12, 14 and 15 of the period) and during *E. cyclo-carpum* feeding (days 16–28 of the period) for protozoal enumeration.

To determine microbial N and OM flow to the duodenum a representative sample of ruminal bacteria and protozoa were isolated and analysed for <sup>15</sup>N enrichment, phosphatidyl choline (protozoa), N and OM. Approximately 200 ml of whole ruminal contents were mixed with an equal volume of 0.9% saline and homogenized in a Waring blender (Waring Products Division, New Hartford, CT, USA) for two 30 s periods to dislodge particulate-associated bacteria. The homogenate was then strained through four layers of cheesecloth and the bacteria were harvested immediately by centrifugation. The filtrate was centrifuged at 800 g, 4°C, for 15 min to remove feed particles and protozoa and then the supernatant was centrifuged at 20 000 g, 4°C, for 45 min to obtain the bacterial pellet. The bacterial pellet was transferred to a container using a minimum amount of deionized water and freeze-dried.

Another 200 ml of the ruminal contents were strained through one layer of cheesecloth and the volume of the filtrate measured. An equal volume of 2% formol saline was added to the feed particles, the particles were mixed, re-restrained and the two liquid fractions were combined for sedimentation of the protozoa. The liquid fraction was poured into a separatory funnel, allowed to stand for 45 min and then the sedimented protozoal layer was collected. The protozoal pellet was washed with 100 ml chilled (4°C) 0.9% saline and centrifuged at 500 g, 4°C for 5 min. The supernatant was poured off and the protozoal pellet was washed two more times with a 100 ml volume of chilled saline followed by centrifugation. The washed protozoal pellet was then transferred to a container using a minimum amount of deionized water and freeze-dried. The dry samples were ground using a mortar and pestle, combined by sheep within period and stored frozen until analysis.

#### Chemical analysis

Analytical DM was determined by placing samples in an oven at 135°C for 2 h (Association of Official Analytical Chemists<sup>26</sup> method 930.05) followed by hot weighing. The OM content was calculated as the difference between 1000 and the ash content (g/kg DM; Association of Official Analytical Chemists<sup>26</sup> method 942.05). The NDF was determined as described by Van Soest *et al.*<sup>27</sup> using heat-stable amylase and sodium sulphite, and ADF was determined according to the Association of Official Analytical Chemists<sup>26</sup> (method 973.18) with modifications to each procedure for use in an ANKOM Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA) and without correction for the ash content.

Samples ground through a 1 mm sieve were further ground using a ball mill (Mixer Mill MM2000; Retsch, Haan, Germany) to a fine powder for measurement of N and <sup>15</sup>N enrichment. Nitrogen was quantified by flash combustion with GC and thermal conductivity detection (Carlo Erba Instruments, Milan, Italy). Crude protein of the feed was calculated as N × 6.25. <sup>15</sup>N enrichment (atom % excess) was measured in feed, duodenal fractions, bacteria and protozoa by flash combustion with isotope ratio MS (VG Isotech, Middlewich, UK). Phosphatidylcholine in the duodenal fractions and protozoa was determined according to Neill *et al.*<sup>28</sup>. The concentrations of the digesta flow markers (Yb and Co) were determined by inductively coupled plasma emission spectrometry (SpectroCiros CCD; Spectro Analytical Instruments, Kleve, KG, Germany).

Ciliate protozoa in preserved ruminal fluid samples were counted using a Neubauer Improved Bright-Line counting cell (0.1 mm depth; Hauser Scientific, Horsham, PA, USA) and a light microscope. VFA in ruminal fluid were quantified using crotonic acid as an internal standard and GC (Model 5890; Hewlett Packard, Little Falls, DE, USA) with a capillary column (30 m × 0.25 mm internal diameter, 1 μm phase thickness, bonded PEG, Supelco Nukol; Sigma-Aldrich Canada, Oakville, ON, Canada) and flame ionization detection. Ruminal and duodenal fluid were analysed for ammonia N concentration by the salicylate–nitroprusside–hypochlorite method using a flow injection analyser<sup>29</sup>. Ruminal pH, protozoal counts (total and species), VFA and ammonia N were averaged for the samples collected at the six different time-points over days 24–28 for each sheep for statistical analysis.

#### Calculations and statistical analysis

Nutrient intakes were calculated for each sheep as the difference between the amount of diet DM offered and refused on days 16–28 and the chemical composition of the diet DM offered and refused for the corresponding period. Nutrient flow to the proximal duodenum was determined by mathematical reconstitution of the duodenal digesta based on the Yb and Co marker concentrations and the nutrient content of the solid and liquid fractions<sup>22</sup>.

The flow of protozoal N to the duodenum was calculated for each sheep by subtracting the mean duodenal flow of phosphatidylcholine measured in the FF group of sheep from the flow in each of the faunated sheep to correct for the flow of feed and endogenous phosphatidylcholine. The corrected duodenal flow of phosphatidylcholine that represented the flow of phosphatidylcholine from protozoa was then multiplied by the ratio of N to phosphatidylcholine concentration in the protozoa isolated from each of the faunated sheep.

The flow of bacterial N was calculated by correcting the N flow to the duodenum and <sup>15</sup>N enrichment in duodenal contents by subtracting the protozoal N flow and <sup>15</sup>N enrichment in ruminal protozoa, respectively. Bacterial N flow to the small intestine was then computed by multiplying the corrected duodenal N flow by the ratio of the corrected <sup>15</sup>N enrichment in duodenal contents and the <sup>15</sup>N enrichment (atomic % excess) in ruminal bacteria.

Nutrient output in faeces was calculated by dividing the daily amount of Yb intake by Yb concentration in the faeces

to determine DM output and then multiplying the DM output by the nutrient concentration of the faecal DM. Recovery of the Yb marker in faeces averaged 96 (SD 3) % and data were not corrected for recovery of the marker. Apparent digestibility of OM, ADF and NDF were calculated as the difference between the nutrient intakes and the amounts passing the duodenum or excreted in faeces.

The data for ruminal fermentation, microbial N flow and nutrient digestibility were analysed as a split-plot design using a mixed linear model (release 9.1; SAS Institute Inc., Cary, NC, USA) with sheep as a random effect, fauna as a fixed effect, *E. cyclocarpum* treatment and the *E. cyclocarpum* treatment  $\times$  fauna interaction as fixed effects, and using sheep  $\times$  fauna in the random statement. The control diet was fed in the first period and the diet supplemented with *E. cyclocarpum* was fed in the second period of the experiment, thus, diet is confounded with time. Least squares means were calculated for each treatment and when the main effect of fauna and the *E. cyclocarpum*  $\times$  fauna interaction were significant the Bonferroni's test was used to evaluate differences among means. The data for the daily protozoal counts when *E. cyclocarpum* was fed were analysed as mixed linear model with sheep as a random effect, fauna as a fixed effect, time as a fixed effect and repeated measure, fauna  $\times$  time interaction and using sheep  $\times$  fauna as the subject. The variance and covariance error structure were estimated using the variance components and compound symmetry structure based on the lowest Akaike's information criterion. When the main effect of time was significant, Dunnett's test was used to compare the daily protozoal numbers with protozoal numbers prior to feeding *E. cyclocarpum* (the average of the counts measured on 3 d prior to feeding *E. cyclocarpum*). Differences among treatments were declared significant at  $P < 0.05$  and trends were discussed at  $P < 0.10$ .

## Results

### Protozoal cell numbers and ruminal fermentation characteristics

There were no protozoa observed in ruminal fluid of any of the sheep of the FF group and only *Entodinium* were observed in sheep of the EN group. Protozoal cell numbers were reduced ( $P < 0.05$ ; Fig. 2) in ruminal fluid 2 h after feeding the diet supplemented with the foliage of *E. cyclocarpum* from the third to the twelfth day of offering in sheep of the TF group, but protozoal numbers were only reduced from the third to the eighth day in sheep faunated with EN. The reduction in protozoal numbers relative to the numbers prior to feeding *E. cyclocarpum* ranged from 31 to 88% in sheep with TF and from 38 to 82% in sheep with EN. When protozoal numbers were expressed as the average of the six samples collected at various times throughout the 24 h feeding cycle from the ninth to the twelfth day of *E. cyclocarpum* supplementation, TF and EN cell numbers tended to be reduced ( $P < 0.09$ ; Table 2) by approximately 25% in sheep fed the diet supplemented with *E. cyclocarpum* compared to sheep fed the control diet.

Total protozoal cell numbers were lower ( $P < 0.05$ ; Table 2) in sheep faunated with TF compared to sheep monofaunated with EN. In sheep faunated with TF, *Entodinia* spp. accounted

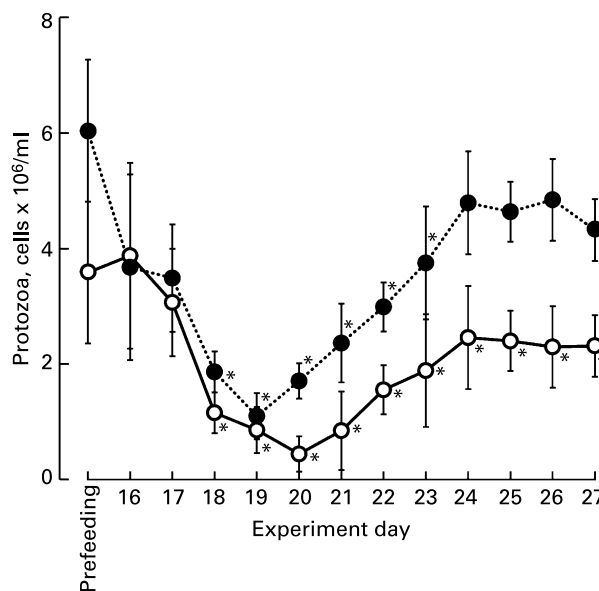


Fig. 2. Protozoal cell numbers in ruminal fluid of sheep faunated with total mixed fauna (○) and *Entodinium caudatum* as a single-species monofauna (●) 2 h after feeding *Enterolobium cyclocarpum*. *E. cyclocarpum* was fed from days 16 to 28 of period 2 (for 12 d in total). Values are means with their standard errors depicted by vertical bars ( $n = 5$ ). Prefeeding cell numbers were determined as the average of cell numbers determined on experiment days 12, 14 and 15 for sheep faunated with each protozoal community. Mean values were significantly different from those of the prefeeding: \* $P < 0.05$ .

for 94–96% of the fauna, *Polyplastron* spp. accounted for 3–6% and *Isotricha* spp. accounted for less than 1%.

There was a significant interaction between *E. cyclocarpum* and fauna for ruminal pH ( $P < 0.05$ ; Table 2), such that there was no effect of the diet on ruminal pH in the FF sheep, but ruminal pH was higher for sheep faunated with TF and EN when fed *E. cyclocarpum* compared to the control diet.

Total VFA concentration in ruminal fluid was higher ( $P < 0.05$ ; Table 2) in sheep fed the diet supplemented with *E. cyclocarpum* compared to that of sheep fed the control diet. There was no effect ( $P > 0.10$ ) of feeding *E. cyclocarpum* on the proportion of acetic acid in ruminal fluid, and although there was a lower ( $P < 0.05$ ) proportion of propionic acid compared to that in sheep fed the control diet, there was no effect ( $P > 0.10$ ) of the dietary treatments on the acetic to propionic acid ratio. There was also no effect ( $P > 0.10$ ) of diet on butyric acid, but the proportion of branched chain and longer acids was higher ( $P < 0.05$ ) in ruminal fluid of sheep fed the diet supplemented with *E. cyclocarpum*.

Fauna had no effect ( $P > 0.10$ ; Table 2) on total VFA concentration, however, the VFA concentration in ruminal fluid of the FF sheep fed the control diet was low (37.5 mM) relative to the faunated sheep fed the same diet (62–66 mM). There was also no effect ( $P > 0.10$ ) of fauna on the proportions of the individual VFA, except for butyric acid. Sheep faunated with the TF had a higher proportion of butyric acid in ruminal fluid than FF sheep ( $P < 0.05$ ).

The ruminal ammonia N concentration was lower ( $P < 0.05$ ; Table 2) in the FF and the faunated sheep fed *E. cyclocarpum* compared to the control diet. The presence of TF increased ( $P < 0.05$ ) ruminal ammonia N concentration above that of

**Table 2.** Effects of feeding *Enterolobium cyclocarpum* on protozoal numbers and ruminal fermentation in sheep with no protozoa (fauna-free, FF), total mixed fauna (TF) or *Entodinium caudatum* as a single-species monofauna (EN)\*

Item	Control			<i>E. cyclocarpum</i>			<i>E. cyclocarpum</i>		Fauna		<i>E. cyclocarpum</i> × Fauna	
	FF	TF	EN	FF	TF	EN	SEM	<i>P</i>	SEM	<i>P</i>	SEM	<i>P</i>
Protozoa (×10 <sup>3</sup> cells/ml)												
Total	–	2957	6671	–	2373	4886	535	0.077	633	0.008	756	0.334
<i>Entodinium</i> spp.	–	2781	6671	–	2290	4886	532	0.086	630	0.007	752	0.298
<i>Polyplastron</i> spp.	–	168	–	–	67	–						
<i>Isotricha</i> spp.	–	7.7	–	–	15.4	–						
pH	6.05	6.03 <sup>a</sup>	6.14 <sup>a</sup>	6.15	6.33 <sup>b</sup>	6.31 <sup>b</sup>	0.04	<0.001	0.06	0.385	0.07	0.038
Total VFA (mm)	37.5	61.6	65.8	87.3	85.7	85.3	4.6	<0.001	5.6	0.225	8.0	0.145
VFA (mol/100 mol)												
Acetic acid	65.2	63.1	64.1	63.6	64.4	65.7	0.9	0.616	1.5	0.858	1.6	0.202
Propionic acid	21.8	19.4	20.8	19.6	17.6	17.7	1.1	0.028	1.7	0.643	1.8	0.821
Butyric acid	8.58	13.14	10.68	11.93	13.22	11.16	0.56	0.170	0.59	0.011†	0.97	0.300
Branched chain and longer acids	4.39	4.32	4.35	4.85	4.77	5.46	0.17	0.007	0.23	0.522	0.292	0.363
Acetate:propionate	3.34	3.80	3.56	3.45	3.87	3.96	0.20	0.239	0.32	0.610	0.35	0.665
Ammonia N (mg N/l)	88.7	152.6	103.3	54.0	88.3	69.1	7.1	<0.001	11.0	0.023†	12.4	0.148

VFA, volatile fatty acids.

<sup>a,b</sup> Mean values within a row, within fauna, with unlike superscript letters were significantly different (*P*<0.05).

\* Protozoal cell numbers and ruminal fermentation characteristics were determined at six different time-points (07.00, 10.00, 12.00, 16.00, 21.00, 01.00 hours) from days 24 to 28 and averaged for each sheep.

† FF ≠ TF, FF = EN, TF = EN (*P*<0.05).

the FF sheep with the ruminal ammonia N concentration in the sheep monofaunated with EN being intermediate.

#### Organic matter and fibre digestibility

Intake of DM and OM did not differ ( $P > 0.05$ ; Table 3) between sheep fed the control diet and the diet supplemented with *E. cyclocarpum*, but because of the high fibre content of the *E. cyclocarpum* (589 g/kg NDF and 486 g/kg ADF), intake of NDF and ADF were about 7 and 24 % higher ( $P < 0.05$ ), respectively, for sheep fed the diet supplemented with *E. cyclocarpum*.

The amount of OM apparently digested in the rumen (corrected for bacterial and protozoal OM) tended ( $P < 0.10$ ) to be lower in sheep fed the diet supplemented with *E. cyclocarpum*, but the amount of NDF and ADF digested was not affected ( $P > 0.10$ ; Table 3). When expressed as the fractional coefficient of digestion, OM digested in the rumen was reduced ( $P < 0.05$ ) from 0.69 in the sheep fed the control diet to 0.64 in sheep fed the diet supplemented with *E. cyclocarpum*. The reduction in the fractional coefficient of OM digestion tended ( $P < 0.10$ ) to be greatest for the sheep with TF. There was also a tendency ( $P < 0.10$ ) for a lower digestion coefficient for ruminal NDF digestion in sheep when fed the diet supplemented with *E. cyclocarpum* compared to the control diet (0.26 v. 0.33).

Fauna had no effect on the ruminal digestion of NDF ( $P > 0.10$ ; Table 3). For ruminal ADF digestion, the *E. cyclocarpum* × fauna interaction was significant ( $P = 0.05$ ), but no differences ( $P > 0.10$ ) were found for the treatment comparisons of interest.

Apparent total tract digestion was reduced ( $P < 0.05$ ; Table 3) for OM and fibre in sheep fed the diet supplemented with *E. cyclocarpum*. There was no effect ( $P > 0.10$ ) of faunation on total tract digestion.

#### Nitrogen intake, intestinal flow and digestion

Nitrogen intake was similar for sheep fed the control and *E. cyclocarpum*-supplemented diet and among the faunated treatment groups ( $P > 0.10$ ; Table 4) as was intended. There was no effect ( $P > 0.10$ ) of diet and fauna on the flow of ammonia N to the duodenum. The flow of non-ammonia N (NAN) to the duodenum was higher in sheep fed the diet supplemented with *E. cyclocarpum* ( $P < 0.05$ ) compared to the control diet (28.7 v. 24.9 g N/d). There were also differences ( $P < 0.05$ ) among the faunated groups of sheep in the flow of NAN, with the flow of NAN highest in the FF sheep, followed by sheep faunated with EN and then lowest in sheep faunated with TF.

The higher flow of NAN in sheep fed the *E. cyclocarpum* treatment compared to the control diet was due in part to a tendency ( $P < 0.10$ ; Table 4) for a higher flow of bacterial N (21.6 v. 20.3 g N/d, respectively). Protozoal N flow accounted for only 6 % of the microbial N flow in the faunated sheep and was not affected ( $P > 0.10$ ) by feeding *E. cyclocarpum*. In addition to being low (0.68 g N/d), protozoal N flow was also quite variable (SEM 0.41 g N/d). Microbial N flow (the sum of the bacterial and protozoal N flow) was also not affected ( $P > 0.10$ ) by supplementing the diet with *E. cyclocarpum*. Bacterial and microbial efficiency, however, were higher ( $P < 0.05$ ) for sheep fed the diet supplemented with *E. cyclocarpum*.

The interaction between *E. cyclocarpum* and fauna for the flow of non-microbial NAN (NMNAN) was significant ( $P < 0.05$ ; Table 4). Feeding the diet supplemented with *E. cyclocarpum* increased the flow of NMNAN in the FF and faunated groups of sheep. However, for sheep fed the control diet, the flow of NMNAN was lower for those faunated with TF and EN compared to the FF sheep, but when fed the diet supplemented with *E. cyclocarpum*, the flow of NMNAN in the sheep with TF and EN increased to the flow observed in the FF sheep. In the absence of protozoa, the flow of NMNAN

**Table 3.** Effects of feeding *Enterolobium cyclocarpum* on intake and apparent digestibility of organic matter (OM) and fibre in sheep with no protozoa (fauna-free, FF), total mixed fauna (TF) or *Entodinium caudatum* as a single-species monofauna (EN)

Item	Control			<i>E. cyclocarpum</i>			<i>E. cyclocarpum</i>		Fauna		<i>E. cyclocarpum</i> × Fauna	
	FF	TF	EN	FF	TF	EN	SEM	<i>P</i>	SEM	<i>P</i>	SEM	<i>P</i>
<b>Intake (g/d)</b>												
DM	1169	1085	1129	1141	1117	1157	19	0.693	23	0.247	33	0.596
OM	1090	1011	1051	1062	1040	1078	18	0.703	21	0.247	30	0.598
NDF	397	373	383	411	400	417	7	0.023	8	0.330	12	0.713
ADF	213	200	206	259	247	262	5	<0.001	6	0.334	9	0.799
<b>Ruminal digestion (g/d)</b>												
OM*	679	730	761	660	648	726	18	0.077	21	0.059†	30	0.559
NDF	110	129	136	108	93	122	13	0.243	20	0.734	23	0.597
ADF	29	61	40	41	36	70	9	0.582	13	0.538	15	0.108
<b>Ruminal digestion (g/g intake)</b>												
OM*	0.623	0.724 <sup>a</sup>	0.728	0.624	0.622 <sup>b</sup>	0.673	0.017	0.005	0.026	0.157	0.030	0.054
NDF	0.276	0.346	0.356	0.269	0.226	0.293	0.034	0.072	0.051	0.765	0.058	0.379
ADF	0.136	0.304	0.198	0.164	0.131	0.268	0.038	0.517	0.056	0.562	0.065	0.050
<b>Total tract digestion (g/g intake)</b>												
OM	0.757	0.751	0.758	0.666	0.662	0.699	0.007	<0.001	0.009	0.245	0.012	0.231
NDF	0.513	0.499	0.530	0.374	0.359	0.434	0.013	<0.001	0.019	0.178	0.023	0.435
ADF	0.461	0.427	0.461	0.286	0.249	0.325	0.017	<0.001	0.023	0.267	0.029	0.661

ADF, acid detergent fibre; NDF, neutral detergent fibre.

<sup>a,b</sup> Mean values within a row, within fauna, with unlike superscript letters were significantly different ( $P < 0.10$ ).

\* Corrected for bacterial and protozoal OM.

† FF = TF, FF ≠ EN, TF = EN ( $P < 0.10$ ).



**Table 4.** Effect of feeding *Enterolobium cyclocarpum* on nitrogen intake, intestinal flow and apparent digestibility in sheep with no protozoa (fauna-free, FF), total mixed fauna (TF) or *Entodinium caudatum* as a single-species monofauna (EN)

Item	Control			<i>E. cyclocarpum</i>			<i>E. cyclocarpum</i>		Fauna		<i>E. cyclocarpum</i> × Fauna	
	FF	TF	EN	FF	TF	EN	SEM	<i>P</i>	SEM	<i>P</i>	SEM	<i>P</i>
N intake (g N/d)	25.7	23.7	24.8	25.2	24.7	25.6	0.43	0.480	0.53	0.228	0.74	0.568
Duodenal N flow (g N/d)												
Ammonia N	0.65	0.71	0.67	0.73	0.58	0.74	0.04	0.849	0.05	0.652	0.06	0.139
NAN	27.9	20.2	24.5	30.3	26.3	27.6	0.52	<0.001	0.63	<0.001†	0.89	0.116
Bacterial N	22.0	17.6	21.5	22.7	20.7	21.4	0.45	0.068	0.55	0.001‡	0.78	0.130
Protozoal N	—	1.08	0.58	—	0.01	1.08	0.29	0.498	0.29	0.503	0.41	0.073
Microbial N	22.0	18.7	22.0	22.7	20.7	22.4	0.53	0.184	0.65	0.012‡	0.92	0.662
NMNAN	5.89 <sup>ac</sup>	1.50 <sup>ad</sup>	2.47 <sup>ad</sup>	7.58 <sup>b</sup>	5.59 <sup>b</sup>	5.20 <sup>b</sup>	0.40	<0.001	0.65	0.008	0.69	0.014
Bacterial efficiency (g N/kg OM ruminally digested)	32.5	24.3	28.2	34.9	31.9	29.7	0.94	0.009	1.15	0.005§	1.63	0.144
Microbial efficiency (g N/kg OM ruminally digested)	32.5	25.9	29.0	34.9	32.0	31.7	1.02	0.024	1.25	0.032§	1.78	0.469
Digestion												
Ruminal* (g N/d)	19.2	21.5	21.7	16.9	18.5	19.7	0.41	<0.001	0.49	0.003§	0.70	0.781
Ruminal* (g N/g intake)	0.746 <sup>ac</sup>	0.911 <sup>ad</sup>	0.877 <sup>acd</sup>	0.672 <sup>b</sup>	0.749 <sup>b</sup>	0.768 <sup>b</sup>	1.51	<0.001	2.47	0.007	0.026	0.012
Total tract (g N/g intake)	0.746	0.734	0.713	0.598	0.586	0.626	0.009	<0.001	0.012	0.737	0.017	0.127

NAN, non-ammonia N; NMNAN, non-microbial non-ammonia N; OM, organic matter.

<sup>a,b</sup> Mean values within a row, within fauna, with unlike superscript letters were significantly different ( $P < 0.05$ ).

<sup>c,d</sup> Mean values within a row, within diet, with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Corrected for bacterial and protozoal N flow.

† FF ≠ TF, FF ≠ EN, TF ≠ EN ( $P < 0.05$ ).

‡ FF ≠ TF, FF = EN, TF = EN ( $P < 0.05$ ).

§ FF ≠ TF, FF ≠ EN, TF = EN ( $P < 0.05$ ).

was increased by 1.69 g N/d when the FF sheep were fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet. The flow of NMNAN was increased to a greater extent, however, when the sheep faunated with TF (4.09 g N/d) and EN (2.73 g N/d) were fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet.

Bacterial and microbial N flow were lowest ( $P < 0.05$ ; Table 4) in the sheep faunated with TF, but did not differ ( $P > 0.10$ ) between the sheep faunated with EN and sheep that were FF. Bacterial and microbial efficiency were higher ( $P < 0.05$ ) in the FF sheep than either of the faunated treatment groups.

The amount of N apparently digested in the rumen (corrected for bacterial and protozoal N) was lower ( $P < 0.05$ ; Table 4) in sheep fed the diet supplemented with *E. cyclocarpum* compared to the diet without supplementation. The amount of N digested in the rumen was also lowest in the FF sheep ( $P < 0.05$ ), however, there was a significant interaction between the dietary and fauna treatments for the coefficient of ruminal N digestion. As was found for the amount of dietary N digested in the rumen, the coefficient of ruminal N digestion was lower for all sheep fed the diet supplemented with *E. cyclocarpum*. When fed the control diet, the coefficient of ruminal N digestion was lower ( $P < 0.05$ ) for the FF sheep compared to the sheep with TF and intermediate for sheep faunated with EN. There were, however, no differences among the faunated groups of sheep fed the *E. cyclocarpum*. Therefore, in the absence of protozoa, the coefficient of ruminal N digestion was reduced by 10% when the FF sheep were fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet. The coefficient of ruminal N digestion was reduced to a greater extent, however, when

sheep faunated with TF (18%) and EN (12%) were fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet. As was found for the ruminal digestion of N, total tract digestion was lower ( $P < 0.05$ ) for sheep fed *E. cyclocarpum*, but there was no effect ( $P > 0.10$ ) of fauna on total tract N digestion.

## Discussion

The antiprotozoal effect of *E. cyclocarpum* was first observed in *in vitro* studies as a reduction in protozoal numbers and bacteriolytic activity and this led to interest in its potential benefits for manipulating digestion and performance of ruminant livestock<sup>1,30</sup>. In *in vivo* studies with sheep and buffalo fed poor-quality forages supplemented with *E. cyclocarpum*, a reduction in ruminal fauna was observed when the foliage (10–34% of diet DM) was fed for periods of short duration<sup>1,16</sup>. In the present experiment, the supplementation of *E. cyclocarpum* (16% of diet DM) to a higher-quality barley-based diet was also effective in reducing total protozoal numbers. Protozoal numbers were reduced from 31 to 88% 2 h after feeding *E. cyclocarpum* and by an average of 25% over the 24 h feeding cycle when measured up to the twelfth day of supplementation. The present results are consistent with a previous experiment during which the same batch of material was fed to sheep fed a similar barley-based diet and with no prior exposure to *E. cyclocarpum*<sup>12</sup>. In that study, protozoal numbers were reduced by 49 to 75% 2 h after feeding the foliage<sup>12</sup>. However, the antiprotozoal effect dissipated and protozoal numbers returned to pre-supplementation or control levels when *E. cyclocarpum* was fed for more than 12–14 d<sup>1,12</sup>. The short-term antiprotozoal action of *E. cyclocarpum* and

some other saponin-containing plants (e.g. *Sesbania sesban*) is attributed to the adaptation of the bacterial community to degrade the saponin<sup>31</sup> and possibly by inactivation of the saponin by salivary proteins<sup>30</sup>. Despite the short-term action of *E. cyclocarpum*, protozoal numbers were reduced in the present experiment by feeding *E. cyclocarpum*, which permitted the assessment of whether such reduced fauna could provide some of the same benefits as defaunation in improving the supply of bacterial N and efficiency of bacterial N synthesis in ruminants fed diets that are otherwise favourable for the growth of protozoa.

Saponins are recognized for their antiprotozoal action, but in addition, saponins have also been reported to inhibit ruminal bacteria and fungi. The inclusion of steroidal saponins from *Yucca schidigera* extract in the medium of pure cultures inhibited cellulolytic ruminal bacteria and fungi but had variable effects on non-cellulolytic bacteria<sup>32,33</sup>. It was suggested that the differential responses of bacterial species to steroidal saponins lies in the structure of the cell membrane, with gram-positive bacteria more susceptible than gram-negative bacteria to inhibitory effects, with the implication that the effects of saponins may be beneficial in much the same way as ionophores<sup>32</sup>. An inhibition of ruminal bacterial synthesis *in vivo*, however, could negate the beneficial effects of a reduced protozoal community on bacterial recycling and bacterial protein supply. In sheep dosed intraruminally with an alfalfa saponin extract, bacterial N flow and efficiency of bacterial N synthesis were decreased<sup>34</sup>. In contrast, the feeding or administration of other saponin-containing plants or plant extracts have increased bacterial numbers and/or the supply of bacterial protein. Total and cellulolytic bacterial numbers in sheep fed *S. sesban* were increased when measured concomitantly with the reduction in protozoal numbers<sup>31</sup>. In the present experiment, bacterial N flow in both the FF and faunated sheep tended to be higher when fed the diet supplemented with *E. cyclocarpum*, indicating that bacterial biomass production was not negatively affected by the saponins.

The total saponin content of the *E. cyclocarpum* leaves used in the present study was determined semi-quantitatively by expressing the haemolytic activity of the saponin extract relative to the activity of digitonin<sup>21</sup>. Haemolytic assays have been used for decades to detect and quantify saponins, however, the haemolytic activity can vary depending on the structure of the saponin and the assay technique used<sup>35</sup>. Based on the haemolytic assay used in the present study, the active saponin content of the *E. cyclocarpum* leaves was 0.8 mg/g DM. This equated to 0.13 g saponins/kg DM for the *E. cyclocarpum* dietary treatment. The dietary concentration of saponins fed in the present study was low relative to other studies that have reported the saponin content of diets supplemented with *E. cyclocarpum* when determined using alternative methods. Hess *et al.*<sup>36</sup> reported the crude saponin content of the fruit of *E. cyclocarpum* to be 19 mg/g DM based on a method of extraction and gravimetric analysis. Navas-Camacho *et al.*<sup>15</sup> reported feeding sheep 1.2 and 4% of diet DM as saponins from *E. cyclocarpum* leaves (approximately 125 mg saponins/g *E. cyclocarpum* DM); the method of analysis, however, was not reported. Few studies have reported the saponin content of *E. cyclocarpum* and differences in methods of quantifying saponins make it difficult to draw conclusions regarding

an effective dietary concentration of saponins from *E. cyclocarpum*.

The responses affecting protein nutrition that are often observed in defaunated ruminants include lower ruminal ammonia N concentration, increased flow of NAN and bacterial N to the intestine, and increased efficiency of microbial protein synthesis in the rumen<sup>8,37</sup>. Improvement in the supply of NAN to the intestine of defaunated ruminants is due to a reduction in protozoal predation of bacteria, a reduction in intraruminal microbial N recycling, an increase in the size of the bacterial N pool and an increase in the net flow of bacterial N to the duodenum<sup>7,38–40</sup>. In addition, defaunation often decreases ruminal degradation of dietary protein and increases the flow of NMNAN to the duodenum<sup>11,40,41</sup>. The supplementation of the barley-based diet with *E. cyclocarpum* and the 25% reduction in ruminal ciliate protozoal numbers increased the flow of NAN from the rumen to the duodenum by 16% compared to the flow in sheep fed the control diet. The increased flow of NAN was due to an increase in the flow of both bacterial N and NMNAN. In addition, efficiency of bacterial protein synthesis in the rumen was improved by 13%.

Feeding a diet supplemented with *E. cyclocarpum* improved NAN and bacterial N flow and the efficiency of bacterial protein synthesis in the rumen, but it also reduced ruminal and total tract digestion of OM and N. Supplementation of the diet with *E. cyclocarpum* increased the proportion of NDF and ADF in diet and could have affected cellulolytic bacterial numbers resulting in the reduction of OM and fibre digestion. The increase in the flow of NMNAN in the FF sheep fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet suggested that the protein of *E. cyclocarpum* was less ruminally digestible than that of the barley silage, barley grain and soyabean meal that it replaced. However, the increase in the flow of NMNAN was even greater in the TF and EN groups of sheep fed the diet supplemented with *E. cyclocarpum* compared to when fed the control diet and indicated that the reduction in ruminal N digestion and increase in flow of NMNAN was largely attributed to the reduction in protozoal numbers and the associated beneficial reduction in ruminal proteolysis<sup>8</sup>.

In addition to saponins, *E. cyclocarpum* may also contain condensed tannins which can range in concentration from 0 to 38 g/kg DM<sup>42,43</sup>. Dietary concentrations of condensed tannins as low as 20–45 g/kg diet DM can form complexes with dietary proteins and reduce protein digestibility<sup>44</sup>. The estimated dietary concentration of tannins fed in the present study may have ranged from 0 to 6.3 g/kg DM, and although the dietary concentration was considered low, the concentration of tannins present in *E. cyclocarpum* could have affected the digestion of the protein of *E. cyclocarpum*. When *E. cyclocarpum* with no condensed tannins was incubated *in sacco* in goats, fractional N disappearance at 24 h of incubation was extensive at 0.92<sup>42</sup>. However, when *E. cyclocarpum* containing 30 g/kg DM was incubated *in sacco*, N disappearance at 48 h was 0.52 in sheep fed *E. cyclocarpum* at 10% of diet DM and was slightly lower at 0.40 in sheep fed *E. cyclocarpum* at 34% of diet DM<sup>16</sup>. In the present study, the lower ruminal and total tract N digestion observed in sheep fed the diet supplemented with *E. cyclocarpum* may be due in part to the presence of tannins and the reduction in the

digestion of the protein of *E. cyclocarpum*. Thus the effect of feeding a diet supplemented with *E. cyclocarpum* on diet digestibility would appear to be due to its nutritive and anti-nutritive composition in addition to its antiprotozoal properties. The design of this experiment permitted separation of the nutritive effects from the antiprotozoal effects, but it may be advantageous to avoid the overlap of these effects by feeding extracts of the potential antiprotozoal compounds.

The number of protozoa in ruminal fluid of the sheep monofaunated with EN was more than double the number in sheep faunated with the TF wherein *Entodinium* spp. account for 94–96% of the total protozoal community. Higher numbers of protozoa in animals monofaunated with *Entodinia* spp. than in those faunated with TF have also been observed in other studies<sup>11</sup>. The present results may suggest that there is a considerable amount of predation of *E. caudatum* in a mixed protozoal community, and perhaps *E. caudatum* is less competitive in a mixed protozoal community where individual species of protozoa are influenced by other species that are present<sup>9</sup>.

In the present experiment, the flow of NAN to the intestine of FF sheep was 25% higher than in sheep faunated with TF and 12% higher than in the sheep monofaunated with EN. The flow of bacterial N to the small intestine of the FF sheep was 17% higher than in sheep faunated with TF, but was only 4.3% higher (numerically) than in sheep faunated with EN. The similarity of the bacterial N flow between the FF sheep and sheep monofaunated with EN was unexpected and in contrast to the results of previous studies<sup>11,17</sup>. In the previous study using the same sheep under similar experimental conditions, bacterial N flow was 22% higher in the FF sheep compared to the sheep monofaunated with EN<sup>17</sup>. The sheep in each of these studies were fed a similar barley-based diet, except that the diet in the present experiment was higher in crude protein due to the additional soyabean meal or *E. cyclocarpum*. In a study with sheep fed haycrop and maize silage-based diets, bacterial N flow was increased by approximately 32 and 85%, respectively, in sheep monofaunated with either *Entodinium* spp. or TF when compared to FF sheep<sup>11</sup>. Differences in the size distribution of particles of insoluble protein and starch among the diets fed in these studies may contribute to the differential effects of the *Entodinium* monofauna on bacterial N flow. The rate of engulfment of bacteria by protozoa can be reduced by the uptake of particulate materials, especially starch grains<sup>45</sup>. At higher concentrations of starch, the engulfment of starch grains reduced the engulfment of bacterial cells by *E. caudatum*<sup>45</sup>. Engulfment by *E. caudatum* is not selective and *E. caudatum* will engulf all particles of the appropriate size, whereas other protozoal species may be more selective towards bacteria<sup>45</sup>. Thus, in the present experiment, despite a larger protozoal community in the sheep monofaunated with EN, the negative effect of protozoa on the NAN and bacterial N supply was greater for the sheep faunated with TF than those monofaunated with EN. In the study of Ivan *et al.*<sup>17</sup>, the duodenal flow of NAN was reduced to the same extent in sheep monofaunated with *Entodinia* spp. or *Polyplastron* spp. compared to FF sheep. In *in vitro* studies, large protozoa isolated from the Type A population engulfed mixed and pure bacterial suspensions at a faster

rate than *Entodinium* spp.<sup>14</sup>, although *in vivo* in a mixed Type A population, the numbers of large protozoa would be considerably lower than *Entodinium* spp. The above combined results suggest that *Entodinia* spp. may not have the greatest negative impact on bacterial N synthesis in a mixed protozoal community.

Protozoal N flow did not differ between the two faunated groups of sheep and accounted for less than 6% of the microbial N flow. Protozoal N may account for 35–65% of the microbial N pool in the rumen but because protozoa are largely retained within the rumen and undergo extensive intraruminal recycling<sup>46</sup>, protozoal N flow to the intestine generally ranges from only 4 to 40% of the total microbial N flow<sup>46–49</sup>. As was found for bacterial N flow, the flow of microbial N (bacterial N plus protozoal N) was 14% higher in the FF sheep than in sheep faunated with the TF, but it did not differ from sheep faunated with EN.

Defaunation can decrease OM and fibre digestibility in the rumen and total tract<sup>37</sup>. A decrease in ruminal OM digestion caused by defaunation can negate the positive effect of an increased intestinal flow of bacterial N on the improvement of bacterial or microbial efficiency. In the present experiment, there were no differences between the FF sheep and sheep faunated with TF for apparent ruminal and total tract OM digestibility, although there was a tendency for a lower amount of OM digestion in the FF sheep compared to the sheep monofaunated with EN. The coefficients of OM digestion, however, were similar for the FF sheep and the sheep monofaunated with EN. This is in agreement with results of a similar study, wherein Ivan *et al.*<sup>17</sup> found no differences in apparent ruminal and total tract OM and fibre digestion between FF sheep and sheep monofaunated with EN. Total VFA concentration was also not affected by the type of fauna. Acetate and butyrate are the major fermentation end products of protozoa<sup>50</sup>, but in the present experiment, there were no differences in the proportions of individual VFA, except for butyrate concentration which was higher in the sheep faunated with TF than in the FF sheep. Thus, with no reduction in OM fermentation, the higher intestinal flow of bacterial N resulted in greater bacterial and microbial efficiency in the FF sheep than in sheep faunated with TF. Many studies have reported that defaunation improved microbial N flow and efficiency<sup>8,37</sup>, however, not all have quantified the contribution of protozoal N to the total microbial N flow to the intestine. Phosphatidylcholine was used as a protozoal marker, but background levels of phosphatidylcholine can be quite high and variable, and the method is unable to quantify low protozoal flows<sup>17</sup>. To assess accurately the implications of defaunation on microbial N supply and efficiency, accurate and sensitive methods will need to be developed to determine protozoal N flow<sup>51</sup>.

The supplementation of a barley-based diet with *E. cyclocarpum* reduced ruminal ciliate protozoal numbers by 25%, increased the intestinal supply of NAN and bacterial N, and improved the efficiency of bacterial protein synthesis. The antiprotozoal effect of dietary *E. cyclocarpum*, however, is of short-term duration, and therefore *E. cyclocarpum* has limited usefulness as an antiprotozoal agent in ruminant diets. However, the antiprotozoal effects achieved in the present study suggest that a 25% reduction in protozoal numbers is

sufficient to achieve the beneficial effects of reduced fauna on N utilization in ruminant production.

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### References

- Leng RA, Bird SH, Klieve A, Choo BS, Ball FM, Asefa G, Brumby P, Mudgal VD, Chaudhry UB, Haryono SU & Hendratno N (1992) The potential for tree forage supplements to manipulate rumen protozoa to enhance protein to energy ratios in ruminants fed on poor quality forages. In *Legume Trees and Other Fodder Trees as Protein Sources for Livestock. Animal Production and Health Paper* 102, pp. 177–191 [A Speedy and PL Pugliese, editors]. Rome: FAO.
- Wallace RJ (2004) Antimicrobial properties of plant secondary metabolites. *Proc Nutr Soc* **63**, 621–629.
- Wina E, Muetzel S & Becker K (2005) The impact of saponins or saponin-containing plant materials on ruminant production – a review. *J Agric Food Chem* **53**, 8093–8105.
- Francis G, Kerem Z, Makkar HPS & Becker K (2002) The biological action of saponins in animal systems: a review. *Br J Nutr* **88**, 587–605.
- Clark JH, Klusmeyer TH & Cameron MR (1992) Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *J Dairy Sci* **75**, 2304–2323.
- Leng RA & Nolan JV (1984) Nitrogen metabolism in the rumen. *J Dairy Sci* **67**, 1072–1089.
- Koenig KM, Newbold CJ, McIntosh FM & Rode LM (2000) Effects of protozoa on bacterial nitrogen recycling in the rumen. *J Anim Sci* **78**, 2431–2445.
- Jouany JP (1996) Effect of rumen protozoa on nitrogen utilization by ruminants. *J Nutr* **126**, 1335S–1346S.
- Williams AG & Coleman GS (1997) The rumen protozoa. In *The Rumen Microbial Ecosystem*, pp. 73–139 [PN Hobson and CS Stewart, editors]. London: Chapman and Hall.
- Jouany JP, Ivan M, Papon Y & Lassalas B (1992) Effects of *Iso-tricha*, *Eudiplodinium*, *Epidinium* + *Entodinium* and a mixed population of rumen protozoa on the *in vitro* degradation of fish meal, soybean meal and casein. *Can J Anim Sci* **72**, 871–880.
- Ivan M, Neill L, Forster R, Alimon R, Rode LM & Entz T (2000) Effects of *Iso-tricha*, *Dasytricha*, *Entodinium*, and total fauna on ruminal fermentation and duodenal flow in wethers fed different diets. *J Dairy Sci* **83**, 776–787.
- Ivan M, Koenig KM, Teferedegne B, Newbold CJ, Entz T, Rode LM & Ibrahim M (2004) Effects of dietary *Enterolobium cyclo-carpum* foliage on the population dynamics of rumen ciliate protozoa in sheep. *Small Rum Res* **52**, 81–91.
- Hristov AN, Ivan M, Rode LM & McAllister TA (2001) Fermentation characteristics and ruminal ciliate protozoal populations in cattle fed medium- or high-concentrate barley-based diets. *J Anim Sci* **79**, 515–524.
- Coleman GS & Sandford DC (1979) The engulfment and digestion of mixed rumen bacteria and individual bacterial species by single and mixed species of rumen ciliate protozoa grown *in vivo*. *J Agric Sci (Camb)* **92**, 729–742.
- Wallace RJ & McPherson CA (1987) Factors affecting the rate of breakdown of bacterial protein in rumen fluid. *Br J Nutr* **58**, 313–323.
- Navas-Camacho A, Laredo MA, Cuesta A, Anzola H & Leon JC (1993) Effect of supplementation with a tree legume forage on rumen function. *Livest Res Rural Dev* **5**, 58–71.
- Ivan M, Koenig KM, Morgavi DP, Rode LM, Newbold CJ & Entz T (2006) Duodenal flow and digestibility in fauna-free sheep and in sheep monofaunated with *Entodinium caudatum* or *Polyplastron multivesiculatum*. *Br J Nutr* **95**, 469–476.
- Eadie JM (1962) Inter-relationship between certain rumen ciliate protozoa. *J Gen Microbiol* **29**, 579–588.
- Canadian Council on Animal Care (1993) *Guide to the Care and Use of Experimental Animals*: 2nd ed., vol. 1, [ED Olfert, BM Cross and AA McWilliams, editors]. Ottawa: CCAC.
- National Research Council (1985) *Nutrient Requirements of Sheep*, 6th rev. ed. Washington, DC: National Academy of Sciences.
- Barbosa P, Gross P, Provan GJ & Stermitz FR (1990) Allelochemicals in foliage of unfavoured tree hosts of the gypsy moth, *Lymantira dispar* L.: 2. Seasonal variation of saponins in *Ilex opaca* and identification of saponin containing aglycones. *J Chem Ecol* **16**, 1731–1738.
- Faichney GJ (1975) The use of markers to partition digestion within the gastrointestinal tract of ruminants. In *Digestion and Metabolism in the Ruminant*, pp. 277–291 [IW McDonald and ACI Warner, editors]. Armidale: University of New England Publishing Unit.
- Siddons RC, Paradine J, Beever DE & Cornell PR (1985) Ytterbium acetate as a particulate-phase digesta-flow marker. *Br J Nutr* **54**, 509–519.
- Udén P, Colucci PE & Van Soest PJ (1980) Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. *J Food Agric* **31**, 625–632.
- Ogimoto K & Imai S (1981) *Atlas of Rumen Microbiology*, pp. 158. Tokyo: Japan Scientific Societies Press.
- Association of Official Analytical Chemists (1995) *Official Methods of Analysis*, 16th ed. Arlington, VA: AOAC.
- Van Soest PJ, Robertson JB & Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**, 3583–3597.
- Neill L, Dayrell MdeS, Kramer JKG & Ivan M (1992) Procedure for analysis of phosphatidylcholine as a protozoa marker in ruminants. *Can J Anim Sci* **72**, 717–719.
- Sims GK, Ellsworth TR & Mulvaney RL (1995) Microscale determinations of inorganic nitrogen in water and soil extracts. *Commun Soil Sci Plant Anal* **26**, 303–316.
- Teferedegne B (2000) New perspectives on the use of tropical plants to improve ruminant nutrition. *Proc Nutr Soc* **59**, 209–214.
- Newbold CJ, El Hassan SM, Wang J, Ortega ME & Wallace RJ (1997) Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *Br J Nutr* **78**, 237–249.
- Wallace RJ, Arthaud L & Newbold CJ (1994) Influence of *Yucca shidigera* extract on ruminal ammonia concentrations and ruminal microorganisms. *Appl Environ Microbiol* **60**, 1762–1767.
- Wang Y, McAllister TA, Yanke LJ & Cheeke PR (2000) Effect of steroidal saponin from *Yucca shidigera* extract on ruminal microbes. *J Appl Microbiol* **88**, 887–896.
- Lu CD & Jorgensen NA (1987) Alfalfa saponins affect site and extent of nutrient digestion in ruminants. *J Nutr* **117**, 919–927.
- Sen S, Makkar HPS & Becker K (1998) Alfalfa saponins and their implication in animal nutrition. *J Agric Food Chem* **46**, 131–140.
- Hess HD, Kreuzer M, Díaz TE, Lascano CE, Carulla JE, Soliva CR & Machmüller A (2003) Saponin rich tropical fruits affect



- fermentation and methanogenesis in faunated and defaunated rumen fluid. *Anim Feed Sci Technol* **109**, 79–94.
37. Eugene M, Archimède H & Sauvant D (2004) Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livest Prod Sci* **85**, 81–97.
  38. Demeyer DI & Van Nevel CJ (1979) Effect of defaunation on the metabolism of rumen micro-organisms. *Br J Nutr* **42**, 515–524.
  39. Orpin CG & Letcher AJ (1983) (1983/84) Effect of absence of ciliate protozoa on rumen fluid volume, flow rate and bacterial populations in sheep. *Anim Feed Sci Technol* **10**, 145–153.
  40. Hsu JT, Fahey GC Jr, Berger LL, Mackie RI & Merchen NR (1991) Manipulation of nitrogen digestion by sheep using defaunation and various nitrogen supplementation regimens. *J Anim Sci* **69**, 1290–1299.
  41. Veira DM, Ivan M & Jui PY (1983) Rumen ciliate protozoa: effects of digestion in the stomach of sheep. *J Dairy Sci* **66**, 1015–1022.
  42. Ahn JH, Robertson BM, Elliott R, Gutteridge RC & Ford CW (1989) Quality assessment of tropical browse legumes: tannin content and protein degradation. *Anim Feed Sci Technol* **27**, 147–156.
  43. Monforte-Briceño GE, Sandoval-Castro CA, Ramirez-Avilés L & Capetillo Leal CM (2005) Defaunation capacity of tropical fodder trees: effects of polyethylene glycol and its relationship to *in vitro* gas production. *Anim Feed Sci Technol* **123–124**, 313–327.
  44. Min BR, Barry TN, Attwood GT & McNabb WC (2003) The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Anim Feed Sci Technol* **106**, 3–19.
  45. Coleman GS (1975) The interrelationship between ruminal ciliate protozoa and bacteria. In *Digestion and Metabolism in the Ruminant*, pp. 149–164 [IW McDonald and ACI Warner, editors]. Armidale: University of New England Publishing Unit.
  46. Harrison DG, Beever DE & Osbourn DF (1979) The contribution of protozoa to the protein entering the duodenum of sheep. *Br J Nutr* **41**, 521–527.
  47. Steinhour WD, Stokes MR, Clark JH, Rogers JA & Davis CL (1982) Estimation of the proportion of non-ammonia nitrogen reaching the lower gut of the ruminant derived from bacterial and protozoal nitrogen. *Br J Nutr* **48**, 417–431.
  48. Faichney GJ, Poncet C, Lassalas B, Jouany JP, Millet L, Doré J & Brownlee AG (1997) Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. *Anim Feed Sci Technol* **64**, 193–213.
  49. Robinson PH, Fadel JG & Ivan M (1996) Critical evaluation of diamminopimelic acid and ribonucleic acid as markers to estimate rumen pools and duodenal flows of bacterial and protozoal nitrogen. *Can J Anim Sci* **76**, 587–597.
  50. Jouany JP, Demeyer DI & Grain J (1988) Effect of defaunating the rumen. *Anim Feed Sci Technol* **21**, 229–265.
  51. Sylvester JT, Karnati SKR, Yu Z, Newbold CJ & Firkins JL (2005) Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. *J Dairy Sci* **88**, 2083–2095.