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Original article

Protozoa involved in butyric rather than lactic fermentative pattern during latent acidosis in sheep

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Abstract – We used six ruminally cannulated Texel wethers to study the relative role of protozoa and lactate-metabolizing bacteria in ruminal fermentative patterns during an induced latent acidosis. The sheep were fed an alfalfa hay diet (H) and latent acidosis was induced, following a short transition period of one week, with a grain-rich acidotic diet (W, 60% wheat + 40% alfalfa hay). Ruminal pH, ruminal volatile fatty acids (VFA), lactate and NH_3 concentrations, protozoa and lactate-utilizing bacterial counts, the relative proportions of three main bacteria implicated in lactate metabolism (a lactate-producing species, *Streptococcus bovis*, and two lactate-utilizing species, *Selenomonas ruminantium*, and *Megasphaera elsdenii*) using specific 16S-rRNA-targeting oligonucleotide probes, and lactate dehydrogenase (LDH) activity were determined for both diets. The pH parameters (mean, minimum, maximum, time and area under pH 6.0 and 5.5) measured with the W diet were indicative of a latent (i.e., subacute and maintained) acidosis. However, a butyric rather than lactic latent acidosis was observed in this study. Total ruminal lactate concentration remained at low levels with the acidotic diet (< 4 mmol·L^{-1}), but changes were observed in VFA composition, which was oriented towards butyrate at the expense of acetate ($P < 0.05$), while propionate remained constant. In agreement with the low ruminal lactate concentration, no changes in the proportion of *S. bovis* 16S-rRNA were observed. The lactate-metabolizing bacterial population also remained fairly constant in number, proportion and activity. The increase in butyrate concentration was accompanied by a proliferation of entodiniomorphs ($P < 0.01$). These results suggest that the protozoa limited lactate accumulation and possibly also the decrease in pH during latent acidosis. Experiments with defaunated and faunated sheep could provide further evidence of the role of protozoa in the development of rumen latent acidosis.

acidosis / rumen fermentation / protozoa / bacteria / lactate

1. INTRODUCTION

Acute acidosis in ruminants results from an excessive consumption of readily fermentable carbohydrates (RFC), causing a marked fall in ruminal pH below 5.0 [1]. In such acute cases of this nutritional disorder, lactic acid, as an end product of ruminal

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microbial fermentation, plays a key role [2]. After a first stage of high VFA production at pH > 6, lactate becomes a major fermentative product at a lower pH [3]. As the pH falls, lactate-producing microorganisms (*Streptococcus bovis*) outnumber the lactate-utilizing microorganisms (*Megasphaera elsdenii* and *Selenomonas ruminantium*) [4]. Protozoa also disappear, and bacterial diversity is largely reduced [5]. If the pH continues to fall, lactobacilli replace *S. bovis*, initiating a spiraling effect with excessive lactate accumulation [6].

Acute acidosis, although very well known, is now rare whereas latent acidosis, defined as a subacute and maintained acidosis, is more discrete but affects a greater number of animals. However, latent acidosis is not so well characterized, because of its less drastic decrease in ruminal pH and its more variable fermentative patterns (ranging amounts of lactate, increased proportions of propionate and/or butyrate) [7–9]. This unstable state may reflect the oscillatory behavior of the ruminal microbial population in response to diet-based fermentative changes. Studies of ruminal microbial changes during latent acidosis [8] or adaptation to high concentrate diets [10–13] highlight the importance of equilibrium between lactate-producing and -utilizing microorganisms (both bacteria, protozoa) in the control of pH and lactate concentration.

The objective of the present study was to assess changes in protozoal populations and in bacteria involved in lactate metabolism during induced latent acidosis in sheep to determine their relative roles in ruminal fermentative patterns.

2. MATERIALS AND METHODS

2.1. Animals, diets and experimental design

Texel wethers ($n = 6$, 2 years of age) with an average BW of 58.9 ± 1.1 kg at the start of the experiment were used. Each animal was fitted with a ruminal cannula (i.d. 62 mm) made of polyamide and polyvinyl chloride (Synthesia, Nogent-sur-Marne, France). Surgery was performed in a sterile environment under general anesthesia (Halothane, ICIU Pharma-vétérinaire, Paris, France) and under the responsibility of a licensed veterinarian with specialized training in large animal surgery. Surgical preparation of the wethers was carried out three months before the start of the study, during which time they received a hay-only diet. Throughout the experimental period, the animals were housed in individual stalls (1.00 × 1.50 m) with automatic waterers and individual feed-bunks, and had free access to mineralized salt blocks (Na 38%, Zn 0.9%, Mn 0.075%, Cu 0.15%, I 0.009%, and Co 0.0003%).

For the duration of the study, the animals were fed two equal portions at 0800 and 2000, of two different diets: a forage diet (H diet) composed of 100% chopped alfalfa hay, and a wheat rich diet (W diet) composed of 60% pelleted ground wheat (3-mm screen) and 40% alfalfa hay. The chemical composition of the experimental feeds and diets is given in Table I. The two diets were offered according to the following experimental feeding design: the H diet was fed ad libitum for a 3-week adaptation period (weeks 1 to 3). From week 4, the animals were fed 90% of the maximum ad libitum H diet intake (i.e. 1.1 kg of DM per day) to ensure that the diet was ingested quickly and without refusals. The H diet was offered for two more weeks (weeks 4 and 5). The incorporation of wheat in the ration was then progressive (10%·d^{-1} during 6 days) to reach the final acidotic W diet containing 60% of wheat and 40% of hay (week 6). The animals were maintained on the W diet for one week (week 7). The total duration of the experiment was 7 weeks, with two sampling periods of one week on weeks 5 and 7.

2.2. Ruminal pH, sample collection and ruminal fermentative parameters

Ruminal pH was measured continuously on seven consecutive days during each sampling period [14]. Each wether was fitted

Table I. Chemical composition of experimental feeds and diets (% DM).

Item	Feed		Diet	
	Alfalfa hay	Wheat	H 100% Hay	W 60% Wheat + 40% Hay
Organic matter	91.2	97.2	91.2	94.8
Crude protein	14.7	11.9	14.7	13.0
NDF	45.2	15.8	45.2	27.6
ADF	32.8	2.6	32.8	14.6
Starch	0	67.9	0	40.7

with an indwelling pH probe in the rumen connected to a data logger, which monitored the pH at 5-min intervals. The following parameters were calculated for each day using the pH kinetics obtained with the indwelling probes: mean, minimum and maximum pH, time and area under pH 6.0, and time and area under pH 5.5. The calculation of the area under the pH threshold (6.0 and 5.5) was the difference between these pH thresholds and the pH value multiplied by the time interval (i.e. 5 min). The positive values (negative values reflect pH greater than the set pH threshold) were summed.

During each of the two sampling periods, ruminal liquid samples (250 mL) were taken manually from the ventral bag of the rumen via the cannula using a suction pump and a rigid plastic tube (length 400 mm; i.d. 15 mm), on two sampling times, before (–1 h) and after the morning meal (+3 h), on two sampling days (days 5 and 7), with a one-day interval to avoid any disturbance of ruminal function. The samples were immediately strained through a 250-µm nylon filter and the filtrate was used for analyses.

VFA and NH_3 contents were respectively determined by gas chromatography using 4-methylvaleric acid as the internal standard (GC 8000 gas chromatograph, Carlo Erba Instrument, Milan, Italy) [15], and by a colorimetric method [16] at the two sampling times on 8 mL of filtrate preserved in duplicate by adding 0.8 mL of 5% (vol/vol) orthophosphoric acid. D- and L-lactic acid content was determined using an enzymatic method on a further 8-mL aliquot (D-/L-lactic acid, Boehringer Mannheim 1112821, Roche Diagnostics, Meylan, France).

2.3. Microbiological analysis

Protozoa were counted at the two sampling times using a Dolfuss cell (Elvetec Services, Clermont-Ferrand, France) according to the procedures described by Jouany and Senaud [17], on 9 mL of filtrate preserved at 4 °C with 1 mL of solution made up of 50% glycerol, 49% distilled water, and 1% glutaraldehyde. Counting was performed during the month following the end of the experiment. Protozoa were further categorized as either entodiniomorphid or isotrichid holotrich ciliates by skeletal plate-staining with Lugol solution.

Lactate-utilizing bacteria were counted for both sampling times by the following counting procedure: decimal dilutions of filtrate in an anaerobic dilution medium [18] were inoculated in triplicate in a lactate culture medium under anaerobic conditions and incubated at 39 °C for 2 weeks at maximum. During incubation, bacterial growth was checked regularly by the turbidity of the medium compared with a non-inoculated control medium. The dilution medium (pH 6.8) contained 85% (vol/vol) distilled water, 7.5% (vol/vol) mineral solution I [18], 7.5% (vol/vol) mineral solution II [18], 0.01% (vol/vol) Resazurine, 0.4% (wt/vol) $NaHCO_3$ and 0.05% (wt/vol) cysteine HCl. The lactate culture medium

Table II. Oligonucleotide probes used in this trial.

Target	Reference	Probes	Sequence (5' - 3')	Tw[a] (°C)
All organisms	Pace et al. [21]	S-*-UNIV-1392-a-A-15	ACGGGCGGTGTGT(AG)C	46
Streptococcus bovis	This study		TCTACTAGTGAAGCAATTGCT	46
Selenomonas ruminantium	This study		GACAGTTTCAATCCCATCACGGGGT	52.5
Megasphaera elsdenii	This study		CGGTCCCCTGACGGGGTTAA	56

[a] Tw: temperature used for the stringent washes.

(pH 6.8) contained 45% (vol/vol) distilled water, 40% (vol/vol) clarified rumen fluid, 7.5% (vol/vol) mineral solution I [18], 7.5% (vol/vol) mineral solution II [18], 1% (vol/vol) D-/L-lactic acid (Sigma L1893, Saint-Quentin Fallavier, France), 0.01% (vol/vol) Resazurine, 0.5% (wt/vol) $NaHCO_3$ and 0.05% (wt/vol) cysteine HCl. After incubation, a significant bacterial growth, and the consumption of at least 50% of D-/L-lactic acid in the medium, showed the presence of an efficient lactate-utilizing flora. D-/L-lactic acid content was assayed using an enzymatic method (D-/L-lactic acid, Boehringer Mannheim 1112821, Roche Diagnostics, Meylan, France). The most probable number (MPN) of lactate-utilizing bacteria was determined from McGrady's tables [19].

The 16S-rRNA quantities of three bacterial lactate-metabolizing species, a lactate-producing species, *Streptococcus bovis*, and two lactate-utilizing species, *Selenomonas ruminantium*, and *Megasphaera elsdenii*, were determined (+3 h from morning feeding) from a freeze-dried part of filtrate (100 mL) using specific 16S-rRNA-targeting oligonucleotide probes. Total RNA was extracted from 50 mg of freeze-dried sample, according to Chomczynski and Sacchi [20]. RNA extracts were blotted on nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Saclay, France) using a dot-blot apparatus (Schleicher and Schuell Co., Dassel, Germany). Hybridization was conducted using synthetic HPLC-purified oligonucleotide probes (MWG Biotech, Courtaboeuf, France) 5'end-labeled with ^{32}P using a T4 kinase (QBIOgene, Illkirch, France). Four γ-^{32}P-labeled oligonucleotide probes were used: the S-*-UNIV-1392-a-A-15 probe targeting all organisms, and one probe for each of the three selected species (Tab. II). Except for the "Univ" probe, oligonucleotide probes were designed and validated in the laboratory (Millet, unpublished data) using RNA extracted from pure cultures of *S. bovis* DSM 20480, *M. elsdenii* DSM 20460 and *S. ruminantium* DSM 2872 and RNA extracted from sheep rumen fluid samples inoculated with different concentrations of each bacterial species (10^7 to 10^9 bacteria·mL^{-1} of rumen fluid) alone or in combination. The temperatures used for the stringent washes are also shown in Table II. Total RNA (100, 75 and 50 ng) from pure cultures of *S. bovis* DSM 20480, *M. elsdenii* DSM 20460, *S. ruminantium* DSM 2872 and 16S-rRNA from *E. coli* (universal standard, Roche Diagnostics, Meylan, France) were used as controls. Radioactivity associated with hybridization signals was detected with a STORM instrument (Molecular Dynamics, Bondoufle, France). Bound probe was quantified by Image Quant software (Molecular Dynamics). The proportions of each species 16S-rRNA

Table III. Daily ruminal pH parameters in sheep fed a control hay diet (H) and a high-wheat diet (W) inducing a latent acidosis ($n = 6$).

Item	Diet		SE	Diet effect
	H	W		
	100% Hay	60% Wheat + 40% Hay		
Mean	6.69	5.85	0.055	***
Minimum	6.41	5.34	0.073	***
Maximum	7.03	6.57	0.034	***
Time under 6.0 (h)	0.0	15.8	0.82	***
Area under 6.0 (pH × h)	0.0	6.0	0.78	**
Time under 5.5 (h)	0.0	3.9	0.83	*
Area under 5.5 (pH × h)	0.0	1.2	0.47	NS

NS: non-significant ($P > 0.1$); † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

were expressed as percentages of the total rRNA signal.

LDH enzyme activity of the ruminal liquid was measured at the two sampling times as follows: 40 mL of filtrate was immediately sonicated (30% duty cycle, 200 W, 4 °C, 16 min divided into eight 2-min periods of sonication with 2-min intervals; Labsonic U, B Braun Biotech Inc, Bethelem, PA, USA). Unbroken cell material was removed by centrifugation (15 000 *g*, 15 min, 4 °C) and the supernatant material was used to estimate LDH activity. Enzyme preparations were stored for one day at –80 °C before assay. LDH activity was measured spectrophotometrically (OD_{340}) (U2000 Spectrophotometer, Hitachi, B. Braun Science Tec, Les Ulis, France) by following the disappearance of NADH at 39 °C. The reaction mixture (3 mL) contained buffer (pH 6.5, triethanolamine-HCl 50 mM, EDTA 5 mM), 7 mM NADH, 8 mM Na-pyruvate, and 15 µL of cell extract. The 8 mM pyruvate concentration had been tested in a pre-experiment as the optimal concentration for measuring LDH activity in these conditions. All assays were performed in triplicate ($n = 3$). The protein content of the enzyme preparations was determined according to Pierce and Suelter [22] using BSA as the standard.

2.4. Statistical analyses

Variance of the data was analyzed at each sampling time using the GLM procedure of SAS [23]. The model was

$$Y_{ij} = \mu + D_i + A_j + E_{ij},$$

where Y_{ij} was the dependent variable for diet i ($i = 1$ to 2), for animal j ($j = 1$ to 6); µ was the population mean for the variable and E_{ij} was a random error associated with the observation ij. In this study, period and diet effects were confounded. However, since the animals were physiologically stable (adult, non-productive), restricted-fed and maintained in a controlled environment, the period effect was assumed to be negligible. The diet effect was tested. Diet means were considered to differ if $P < 0.05$, and a tendency to differ was considered to exist if $0.05 < P < 0.1$.

3. RESULTS

3.1. Ruminal pH and fermentative parameters

The mean, minimum and maximum ruminal pH values were lower with the W than with the H diet ($P < 0.001$; Tab. III). The time under pH 6.0 and under pH 5.5

Table IV. Ruminal VFA composition, lactate and ammonia concentrations in sheep fed a control hay diet (H) and a high-wheat diet (W) inducing a latent acidosis ($n = 6$).

Item	Time (h)/ feeding[a]	Diet		SE	Diet effect[b]
		H 100% Hay	W 60% Wheat + 40% Hay		
Total VFA (mmol·L^{-1})	–1	85.1	102.2	4.99	†
	+3	124.4	122.9	10.40	NS
Acetate (mol %)	–1	70.5	65.0	1.17	*
	+3	70.2	62.7	1.27	**
Propionate (mol %)	–1	16.6	18.5	1.04	NS
	+3	18.8	20.2	1.13	NS
Butyrate (mol %)	–1	7.4	11.7	1.06	*
	+3	6.8	13.0	1.23	*
Isobutyrate (mol %)	–1	1.8	1.2	1.10	*
	+3	1.0	0.9	0.08	NS
Valerate (mol %)	–1	0.9	1.0	0.07	NS
	+3	1.8	1.1	0.07	***
Isovalerate (mol %)	–1	2.5	2.2	0.17	NS
	+3	1.2	1.6	0.14	NS
Caproate (mol %)	–1	0.3	0.4	0.08	NS
	+3	0.2	0.6	0.18	NS
Acetate:propionate ratio	–1	4.3	3.4	0.24	NS
	+3	3.8	3.3	0.20	NS
Acetate:(propionate + butyrate)	–1	2.9	2.2	0.15	*
	+3	2.7	1.9	0.13	**
Total lactate (mmol·L^{-1})	–1	0.84	1.18	0.113	†
	+3	0.39	3.67	2.084	NS
L-Lactate (mmol·L^{-1})	–1	0.41	0.48	0.120	NS
	+3	0.17	1.49	0.877	NS
D-Lactate (mmol·L^{-1})	–1	0.44	0.70	0.069	*
	+3	0.22	2.18	1.208	NS
NH_3 (mmol·L^{-1})	–1	16.4	12.4	1.14	*
	+3	19.5	12.4	1.61	*

[a] Time (h) from morning feeding.
[b] NS: non-significant ($P > 0.1$); † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

increased with the W diet compared with the H diet ($P < 0.001$ and 0.05, respectively), pH remaining for 3.9 h under pH 5.5 and for 15.8 h under pH 6.0 with the W diet vs. 0 h with the H diet. The area under pH 6.0 was higher with the W diet than with the H diet (6.0 vs. 0 pH × h, respectively; $P < 0.01$) whereas the area under pH 5.5 was not significantly affected by the diet.

The total ruminal VFA concentration tended to be higher before feeding with the W diet (102.2 mmol·L^{-1}) than with the H diet (85.1 mmol·L^{-1}) ($P < 0.1$; Tab. IV), but it stayed constant between the two diets after the feeding. The molar proportion of acetate and the acetate:(propionate + butyrate) ratio were reduced with the W diet compared with the H diet, before ($P < 0.05$)

Table V. Ruminal protozoa number in sheep fed a control hay diet (H) and a high-wheat diet (W) inducing a latent acidosis ($n = 6$).

Item	Time (h)/ feeding[a]	Diet		SE	Diet effect[b]
		H 100% Hay	W 60% Wheat + 40% Hay		
Total protozoa[c] ($10^3 \cdot mL^{-1}$)	–1	119 (5.04)	333 (5.45)	24.7	**
	+3	232 (5.31)	345 (5.45)	69.5	NS
Holotrichs ($10^3 \cdot mL^{-1}$)	–1	1.7 (3.16)	9.1 (3.76)	1.56	*
	+3	51.9 (4.56)	70.5 (4.65)	21.62	NS
Entodiniomorphs ($10^3 \cdot mL^{-1}$)	–1	117 (5.03)	323 (5.44)	24.6	**
	+3	180 (5.19)	275 (5.34)	58.6	NS

[a] Time (h) from morning feeding.
[b] NS: non-significant ($P > 0.1$); † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
[c] Values in parentheses are the $\log_{10}$ transformations of the data.

and after feeding ($P < 0.01$). Before and after feeding, the molar proportion of butyrate was higher with the W diet than with the H diet ($P < 0.05$). Molar proportions of propionate, isovalerate and caproate, and the acetate:propionate ratio were not significantly affected by the diet. Compared with the H diet, the molar proportion of isobutyrate decreased before feeding with the W diet ($P < 0.05$), and the molar proportion of valerate decreased after feeding ($P < 0.001$).

The ruminal lactate concentration tended to be higher before feeding with the W diet than with the H diet ($P < 0.1$; Tab. IV). D and L-lactate proportions were approximately 56 and 44%, respectively. The ruminal ammonia concentration was lower with the W diet than with the H diet at both sampling times ($P < 0.05$).

3.2. Microbiological parameters

Compared with the H diet, the total protozoa count numerically increased with the W diet. This diet effect was significant only before feeding ($P < 0.01$; Tab. V) and was mainly due to an increase in the numbers of entodiniomorphs, which made up the great majority (more than 97%) of the protozoal population.

Lactate-utilizing bacterial counts showed a slight but non-significant increase with the W diet compared with the H diet (Tab. VI).

The total rRNA concentration and the relative 16S-rRNA proportion of *S. bovis*, a lactate-producing species, were not significantly modified by the acidotic diet. Concerning the lactate-utilizing species, there was a trend for the relative population size of *S. ruminantium* to be higher with the W diet than with the H diet ($P < 0.1$; Tab. VI), whereas *M. elsdenii* 16S-rRNA was not detected in the rumen of the animals for either diet.

Neither total nor specific LDH activities differed between the diets before feeding (Tab. VII). After feeding these activities tended to be 50% lower with the W diet than with the H diet ($P < 0.1$).

4. DISCUSSION

The modifications of mean ruminal pH, time and area values induced by the W diet agreed with those observed in a previous experiment on sheep fed a similar diet [14]. Several definitions of latent acidosis can be found in the literature, e.g., a mean daily pH

Table VI. Rumen liquid bacterial populations in sheep fed a control hay diet (H) and a high-wheat diet (W) inducing a latent acidosis ($n = 6$).

Item	Time (h)/ feeding[a]	Diet		SE	Diet effect[b]
		H 100% Hay	W 60% Wheat + 40% Hay		
Lactate-utilizing bacteria	–1	178 (7.25)	650 (7.81)	214.0	NS
Number[c] ($10^5 \cdot mL^{-1}$)	+3	239 (7.38)	1583 (8.20)	521.3	NS
Total rRNA ($\mu g \cdot g^{-1}$ DM)	+3	1369	769	301.6	NS
S. bovis rRNA as % of total rRNA	+3	0.12	0.21	0.052	NS
M. elsdenii rRNA as % of total rRNA	+3	ND[d]	ND	–	–
S. ruminantium rRNA as % of total rRNA	+3	0.09	0.75	0.143	†

[a] Time (h) from morning feeding.
[b] NS: non-significant ($P > 0.1$); † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
[c] Values in parentheses are the $\log_{10}$ transformations of the data.
[d] ND: not detected.

Table VII. Rumen liquid lactate dehydrogenase (LDH) activity in sheep fed a control hay diet (H) and a high-wheat diet (W) inducing a latent acidosis ($n = 6$).

Item	Time (h)/ feeding[a]	Diet		SE	Diet effect[b]
		H 100% Hay	W 60% Wheat + 40% Hay		
LDH total activity	–1	0.20	0.22	0.037	NS
(μmol lactate$\cdot min^{-1} \cdot g^{-1}$ DM)	+3	0.15	0.08	0.024	†
LDH specific activity	–1	1.34	1.17	0.226	NS
(μmol lactate$\cdot min^{-1} \cdot g^{-1}$ protein)	+3	1.04	0.57	0.154	†

[a] Time (h) from morning feeding.
[b] NS: non-significant ($P > 0.1$); † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

between 6.25 and 5.5 [24], repeated bouts of depressed rumen pH between 5.2 and 5.6 [25], or between 5.0 and 5.5 [1]. Ruminal pH values observed in our study with the acidotic diet are consistent with these definitions and indicate that the animals were subjected to a latent, i.e. subacute and maintained, acidosis.

Total ruminal lactate concentration remained at low levels with the W diet (< 4 mM). Low concentration of ruminal lactate was also reported in sheep [13, 14] and in steers [7, 26] fed high starch diets (> 40% on a DM basis). The total ruminal VFA concentration tended to increase with the W diet before feeding and stayed constant after feeding. Although nonsystematic [27], the introduction of RFC in the diet generally results in an increase in the total ruminal VFA concentration [4]. A higher post-prandial absorption rate of VFA with the W diet than with the H diet, related to the lower pH,

may explain the similar post-feeding VFA concentrations in the two diets [28]. The ruminal acetate proportion decreased with the W diet, while the propionate proportion was stable, and the butyrate proportion increased. In the literature, digestion of high starch diets gives different VFA fermentative profiles in the rumen. In vivo studies on ruminants fed diets containing 30 to 70% of starch report a decrease in the acetate proportion in favor of propionate [29, 30], butyrate [14, 31, 32], or both [8, 10, 12].

Ruminal NH_3 concentration was lower with the W diet than with the H diet. This is in agreement with other studies on steers in subacute acidosis [8] or feeding an increasing proportion of concentrate [10, 11]. This could be explained by a greater microbial assimilation with the W diet than with the H diet.

The original approach of this work was to explain the butyric rather than lactic fermentative pattern during latent acidosis by changes in the microbial community. It may be supposed that lactate did not accumulate with the W diet because of a low production, or because of a balance between production and utilization by lactate-metabolizing microorganisms, and especially by bacteria. Since *S. bovis*, *S. ruminantium* and *M. elsdenii* are known to be the major bacterial species involved in ruminal lactate metabolism [33], we used specific oligonucleotide probes targeting 16S-rRNA of these bacteria in the rumen. This technique has been largely applied to ruminal cellulolytic bacteria [34–37], but to our knowledge, not to ruminal lactate-metabolizing bacteria. Considering the results obtained after feeding by the probe technique for *S. ruminantium* and those obtained at the same sampling time for lactate-utilizing bacterial counts, we observed that the proportion of 16S-rRNA specific for *S. ruminantium* relative to total rRNA was in agreement with the contribution of lactate-utilizing bacteria within the total ruminal bacterial population, known to range between 10^9 to 10^{10} cells·mL^{-1} [38].

The *S. ruminantium* 16S-rRNA proportion tended to increase with the W diet. However, *S. ruminantium* is not an exclusive lactate-utilizing bacterium and can ferment other substrates if lactate is limiting [39]. *M. elsdenii* 16S-rRNA was not detectable in our study, suggesting that the population levels were below the detection limit of the technique used (approx. 10^6 cells·mL^{-1}). This bacterium is usually responsible for metabolizing 60 to 80% of the lactate produced in the rumen [33]; this lack of detection may simply reflect the low concentration of lactate that could be used as the substrate by this bacterium. Concerning the lactate-producing bacteria, no diet effect was observed on the *S. bovis* 16S-rRNA proportion. Mackie et al. [12] in sheep during the adaptation to a high concentrate diet, and Goad et al. [8] in steers in latent acidosis, observed no ruminal lactate accumulation with an increase of both lactate-producing and -utilizing bacteria populations when using classical enumeration techniques. In both studies, the increase of lactate-producing bacteria concerned *Lactobacillus* but not *S. bovis*. In a more recent study using a real-time Taq nuclease assay technique on high grain-fed steers [40], ruminal lactate did not accumulate either. The *S. bovis* population remained constant, but it was shown that the *M. elsdenii* population increased.

Total and specific LDH activity of liquid associated microorganisms, responsible for the conversion of pyruvate into lactate, was similar before feeding with both diets, and tended to be lower after feeding with the W diet, i.e., when ruminal pH was minimal (5.34). This was surprising since other authors [41] observed in vitro that the LDH activity of pure cultures of *S. bovis* or *S. ruminantium* increased when culture pH decreased. The low LDH activity found with the acidotic diet and the lack of changes observed in the lactate-producing and -utilizing bacteria indicate a low overall lactate production with the acidotic diet.

Compared with the H diet, protozoal numbers were higher with the W diet. This was consistent with the fact that the protozoal population increases with the proportion of starch in the diet, up to a certain level above which the protozoa concentration decreases, sometimes until defaunation [38, 42]. Protozoa can have several effects on ruminal fermentation. Entodiniomorphs, which represented 87% of the total protozoal population in the present study, engulf starch granules very rapidly [43] and thus compete effectively with amylolytic bacteria for their substrate [2]. In addition, starch is fermented by protozoa at a slower rate than in amylolytic bacteria and the main products of fermentation are VFA rather than lactate [42, 44]. Thus they have a stabilizing effect in the rumen by delaying fermentation. Entodiniomorphs are also able to consume lactate and thus may play an essential role in the prevention of lactate accumulation [45, 46]. Moreover, entodiniomorphs could be involved in the fermentative pattern towards butyrate observed with the W diet. Indeed, they ferment starch preferentially to butyrate rather than to propionate [44, 47]. On the contrary to our results, Goad et al. [8] observed a decrease in protozoa numbers in steers during latent acidosis. However, these different observations may result from the different ruminal pH conditions during the studies. Before feeding, the ruminal pH of the acidotic animals was lower in the study of Goad et al. [8] than in the present study (5.5 vs. 6.5, respectively). It can be supposed that the present study describes an earlier stage of latent acidosis and the study of Goad et al. [8] a later stage of latent acidosis.

Bacteria may also be involved in the fermentative pattern towards butyrate. *M. elsdenii* is a major producer of butyrate from lactate [48] but in this experiment, the level of this bacterium was too low to have a significant impact, as suggested by the results obtained with the 16S-rRNA targeted probes technique. *Butyrivibrio fibrisolvens*, a bacterium not examined in our study, is also a major butyrate-producing bacterium in the rumen [39]. It has been shown to be predominant in sheep during adaptation to high concentrate diets [12], and so this species may also contribute to butyrate production in the present study.

5. CONCLUSION

The present study shows that a high starch acidotic diet induced a butyric rather than lactic latent acidosis. An increase in entodiniomorph protozoa numbers may have promoted butyrate production, restricted bacterial access to starch, and so limited ruminal lactate accumulation. It is suggested that protozoa may play a major role in preventing the development of acidosis by the direct and indirect control of ruminal fermentation and bacterial populations. However, other regulation mechanisms within the rumen are possible. The comparison between defaunated and faunated animals would clarify the role of ciliate protozoa during latent acidosis.

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