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The effect of *Saccharomyces cerevisiae* and *Aspergillus oryzae* on fermentations in the rumen of faunated and defaunated sheep; protozoal and probiotic interactions

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Summary — We measured the effect of the direct addition to the rumen of *Saccharomyces cerevisiae* (SC 50 mg/day) and *Aspergillus oryzae* (AO 3 g/day) on the fermentation processes in fistulated sheep. The measurements were carried out on animals whose rumens were first defaunated and then refaunated. The animals received a ration composed of hay (600 g/day), barley (600 g/day) and soybean meal (150 g/day), fed twice daily in two equal meals. The number of fungi and total, viable or cellulolytic bacteria were lower after the inoculation of protozoa in defaunated rumens. The probiotics stimulated the development of total bacteria but reduced the population of cellulolytic bacteria. The addition of the probiotics and the presence of protozoa each incurred a decrease in the redox potential values. The association of both treatments had an additive effect on this parameter. The two probiotics and the protozoa stabilized the rumen pH after the meal, maintaining it above the value of 6 for a longer period of time. The positive effects on pH were accumulated in the refaunated animals receiving probiotics. The ammonia nitrogen concentration was considerably increased by the presence of the protozoa; the probiotics increased the ammonia concentration only in the refaunated sheep. The methane and hydrogen proportions in the fermentation gases were invariably higher in the refaunated animals. The probiotics had no clear effect either on the gas composition or the concentration and the composition of the mixture of volatile fatty acids; only the concentration of isovalerate was significantly increased by probiotics and only in refaunated animals. The protozoa did, however, considerably increase the concentrations of acetate, butyrate and isoacids and decreased the concentration of caproate.

rumen / probiotics / protozoa / fermentation

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Résumé — Effet de *Saccharomyces cerevisiae* et *Aspergillus oryzae* sur les fermentations dans des rumens de moutons faunés et défaunés ; interactions entre les effets des protozoaires et des probiotiques. *Nous avons mesuré les effets de l'ajout direct dans le rumen de* Saccharomyces cerevisiae *(SC 50 mg/jour) et d'*Aspergillus oryzae *(AO 3 g/jour) sur les processus fermentaires au niveau de cet organe chez des moutons dont le rumen était défauné dans un premier temps puis refauné. Les animaux recevaient une ration composée de foin (600 g/jour), d'orge (600 g/jour) et de tourteau de soja (150 g/jour), distribuée en deux repas égaux par jour. L'introduction de protozoaires dans le rumen a entraîné une diminution des bactéries totales, viables, cellulolytiques, et des champignons. La présence des probiotiques a stimulé le développement de la population des bactéries totales mais a provoqué une diminution des effectifs des bactéries cellulolytiques. L'ajout de probiotiques ainsi que la présence des protozoaires ont chacun provoqué une diminution des valeurs du potentiel redox. L'association des deux traitements a eu un effet additif sur ce paramètre. Les deux probiotiques ainsi que les protozoaires ont stabilisé le pH après le repas en le maintenant au-dessus de la valeur 6 pendant un temps plus long. Les effets positifs sur le pH ont été cumulés chez les animaux refaunés traités par les probiotiques. La concentration en azote ammoniacal a été fortement augmentée par la présence de protozoaires ; les probiotiques ont augmenté la concentration en ammoniac uniquement dans le rumen des animaux refaunés. Les proportions de méthane et d'hydrogène dans les gaz de fermentation ont toujours été plus élevées chez les animaux refaunés. Les probiotiques n'ont, en revanche, pas modifié les proportions des différents gaz de fermentation ni eu d'effet sur la concentration des acides gras volatils et la composition du mélange ; seule la concentration en isovalérate a été significativement augmentée par l'ajout de probiotiques et cela n'a été observé que chez les animaux refaunés. Les protozoaires ont, en revanche, fortement augmenté les concentrations en acétate, butyrate, isoacides et diminué la concentration en caproate.*

rumen / probiotique / protozoaire / fermentation

INTRODUCTION

In ruminants, over 50% of the ingested organic matter is digested in the rumen. Plant materials are hydrolyzed in the rumen due to the action of bacteria, protozoa and anaerobic fungi. The intensity of digestion and the products of fermentation determine the supply of nutrients to the animal and affect, therefore, animal production. For this reason, extensive studies have been centered around the control and manipulation of the rumen digestive processes with the aid of additives (Jouany, 1994) over the past 20 years. Probiotics, composed of yeasts or other live aerobic fungi associated with their culture medium, are considered to be natural products and have, in this light, been the object of much interest in recent years as possible food additives. Their use has been associated with reduced digestive disorders in animals being managed under intensive feeding conditions (Wallace and Newbold, 1992). The production responses and digestive effects are, however, generally slight and highly variable.

Several authors have reported that fungal probiotics may stimulate the number of bacteria in the rumen (Wallace and Newbold, 1992). To our knowledge, however, no information is available on their interaction with the rumen protozoa. It is generally acknowledged that protozoa play a role in stabilizing the physicochemical conditions of the rumen medium, explaining their positive effect on cellulolysis in high starch diets (Jouany, 1994). Several studies with AO and SC have also associated fungal probiotics with a stabilizing effect on pH and a decrease in the concentration of lactic acid in the rumen fluid (Martin et al, 1989), which are sometimes associated with enhanced plant cell wall digestion. However, many other protozoal effects are different from those of probiotics. It should be noted, in particular, that the results attributed to the addition of protozoa to a defaunated rumen are fairly reproducible, whereas the effect

of probiotics appears to be more aleatory and more dependant on experimental conditions.

The object of this work was to determine the effect of two probiotics, a yeast belonging to the species *Saccharomyces cerevisiae* and a fungus belonging to the species *Aspergillus oryzae*, on the digestion of a mixed diet composed of equal portions of grass hay and pelleted barley. The "protozoa–probiotic" interactions which form the most original aspect of this work, were studied by comparing the probiotic response measured in defaunated animals to that measured in the same animals following refaunation.

MATERIALS AND METHODS

Animals, feed and experimental design

A total of nine castrated male adult Texel sheep fitted with rumen cannulae and weighing 72.5 ± 5.0 kg, were used (labeled from A to I) in this study. They were defaunated by emptying and washing the rumen according to the method described by Jouany and Sénaud (1979a). The measurements began following a 6 week adaptation period to the defaunated state. Rumen content samples were taken on a regular basis to verify the absence of protozoa within the rumen. The animals were used in this defaunated state during the first two periods of the study. They were subsequently inoculated with 200 ml of rumen contents, containing a mixed fauna composed of the genera *Isotricha* (10^3/ml), *Epidinium* (10^4/mL), *Eudiplodinium* (10^4/mL) and *Entodinium* (10^5/mL) and characterized as belonging to type B according to the Eadie classification system (1962). The third period of measurement began 4 weeks after the stabilization of the ciliate population.

The sheep were housed in individual stalls located in a building specifically designed to avoid any direct contact between individual animals with the resulting contamination risks. The animals received 1 350 g/day of a mixed diet composed of timothy grass hay (44.5%), pelleted barley (44.5%) and soybean meal (11.0% of dry matter [DM] of the ration), fed twice daily in equal meals, at 0900 and 1600 hours. The chemical composition of the ration is detailed in table I. According to the high proportion of barley in this diet, the animals were adapted progressively for 1 month before the begining of the measurements. During each period, the measurements were carried out simultaneously on six animals. Three animals died during the experiment. Animal B died at the end of the second measurement period following necrosis of the liver, aggravated by heparin injections during some blood samplings. Animals E and F died after having undergone two defaunation treatments within the space of 3 months, due to the accidental apparition of ciliates of the genus *Entodinium*. Animals B, E and F were replaced respectively by animals I, G and H.

The two probiotics tested corresponded to a strain of *Saccharomyces cerevisiae* (SC, registered as CNCM I-1096, Institut Pasteur, France) and a strain of *Aspergillus oryzae* (AO). These

Table I. Chemical composition of the diet (g/day/animal).

	Timothy grass hay	*Pelleted barley*	*Soybean meal*	*Total*
Fresh matter	600.0	600.0	150.0	1 350.0
Dry matter	539.2	535.5	133.9	1 208.6
Organic matter	504.7	507.1	124.6	1 136.7
Crude protein (N x 6.25)	45.2	62.0	67.7	174.9
Neutral detergent fiber	387.8	82.9	11.2	481.9
Acid detergent fiber	224.3	30.7	5.8	260.8
Acid detergent lignin	27.4	4.2	0.3	31.9
Starch	–	267.8	–	267.8

two additives, which were composed of living cells (at least 20.10^9 living cells/g for SC) without their culture medium, were provided by the SANTEL[1] company. They were administered in one daily dose through the cannulae.

The experiments were conducted according to the following experimental design:

– Period 1: six defaunated animals (A, B, C, D,E, F) without probiotics,

– Period 2: among the six defaunated animals, three (A, B, C) received SC (50 mg/day); the three others (D, E, F) received AO (3 g/day),

– Period 3: six animals (A, B, C, D, G, H) were refaunated and received no probiotics,

– Period 4: among the six refaunated animals, three (A, I, C) received SC (50 mg/day); the three others (D, G, H) received AO (3 g/day).

Periods 2 and 4 began 2 weeks after the addition of probiotics.

Rumen sampling procedure

Rumen fluid samples were taken through the rumen cannula at different times throughout the day during the first 2 weeks of experiment: T0 (before the meal), 30 min, 1 h, 1 h 30, 2 h, 3 h, 5 h and 7 h after the beginning of the morning meal. The pH and redox potential of the rumen fluid were measured immediately after sampling. The fluid was then filtered through nylon gauze (pore diameter = 250 µm) and aliquots, for the measurement of ammonia nitrogen, volatile fatty acids, protozoa counts and total bacterial cell counts, were taken from the filtrate. Only the mean values of the numbers of protozoa and bacteria throughout the day will be presented in this paper. The sampling of the rumen fluid was repeated for 6 days during each period. Total viable bacteria, cellulolytic bacteria and anaerobic fungi were determined only in the rumen fluid samples taken just before feeding.

Samples of the fermentation gases in the rumen were taken with the aid of special cannulae developed for this type of measurement (Jouany and Sénaud, 1979b), for 4 consecutive days during the third week of the experiment at the following times: T0 (before the meal), 1 h, 2 h, 3 h and 7 h after the beginning of the morning meal.

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The total digesta were collected by completely emptying the rumen 5 hours after the morning meal. The volume, weight and pH of the rumen contents were measured. Aliquots were taken after filtration though a nylon gauze (pore diameter = 250 µm) for the determination of the ammonia nitrogen concentration and volatile fatty acids.

Measurements

Rumen fluid filtrate (1 ml) was added to a solution of 12.5% (p/v) NaCl (6 ml) and stored at –15 °C prior to the measurement of the ammonia nitrogen according to the method of Berthelot, modified and adapted for a Technicon autoanalyzer (Van Eeneame et al, 1969).

A separate aliquot of rumen fluid (5 ml) was added to 5% (v/v) orthophosphoric acid (0.5 ml) and stored at –15 °C prior to volatile fatty acid (VFA) analysis and lactate determination by gas chromatography according to the method described by Jouany (1982). Gases were analyzed by gas chromatography (Jouany and Sénaud, 1978).

The protozoa were counted under a binocular magnifier (x 80) in Dolfuss cells according to the method described by Jouany (1978). The total bacterial cell counts were determined after staining with orange acridine under an epifluorescent microscope (Ben Salah, 1994). The roll tube technique (Bryant, 1972) was used for enumerating total viable bacteria and cellulolytic bacteria; the same technique was adapted for the determination of anaerobic fungi according to Joblin (1981). The total viable bacteria and cellulolytic bacteria numbers were also determined using a most-probable number method (Clarke and Owens, 1983).

Statistical analyses

To rule out as completely as possible any risk of protozoal contamination during the periods corresponding to the "defaunated" state, we first made measurements when all the animals were defaunated; we subsequently introduced a mixture of ciliates into the rumen of all the animals and conducted the two periods, with and without probiotics, during which all animals were faunated.

The data were processed by variance analysis using the GLM procedure of SAS (SAS Insti-

tute Inc, 1987) according to the following model: $Y_{ijk} = m + A_i + F_j + F_j(P_k) + E_{ijk}$, where Y = observed parameter, m = overall mean, A = animal effect, F = fauna effect, $F(P)$ = probiotic effect nested within fauna effect and E = residual. This model, within which the probiotic effect is nested within the fauna effect, is adapted to the experimental design, especially in the case of an existing relationship between fauna and probiotics. This interaction was also assessed with the use of a second model: $Y_{ijk} = m + A_i + F_j + P_k + FP_{jk} + E_{ijk}$, where Y = observed parameter, m = overall mean, A = animal effect, F = fauna effect, P = probiotic effect, FP = interaction between fauna and probiotic and E = residual. As the parameters of rumen fermentations were measured on kinetic curves, we employed the option "repeated measures analysis of variance" which takes into account the nonindependance of the measurements between times of sampling.

Means were compared in pairs using Duncan's multiple range test. All tests were carried out under a bilateral hypothesis with the level of significance at 5%.

RESULTS

Although the presence of the probiotics had no significant effect on the protozoal numbers (table II), there was a tendancy for *Epidinium* and, to a lesser extent, the total ciliates to increase. The probiotics had a positive and significant effect ($P < 0.001$) on the total bacterial cell numbers, which were higher in the presence of SC and much higher in AO-treated animals (table III). However, these effects disappeared for the total viable bacteria and the anaerobic fungi. The numbers of cellulolytic bacteria were significantly lower with SC ($P < 0.05$) and tended to decrease with AO ($P > 0.05$). The presence of protozoa dramatically decreased all the tested bacteria. The total viable bacteria decreased more than three-fold ($P < 0.001$) and the cellulolytic bacteria more than two-fold ($P < 0.05$) after inoculation of protozoa. The anaerobic fungi were eight times less numerous in the presence of protozoa ($P < 0.001$). There was no interaction between protozoa and probiotics on the tested rumen microbial populations.

The redox potential values of the rumen contents (table IV) were low before feeding and then increased, reaching a maximum 5 h after the meal, after which they decreased until the subsequent meal. The presence of either protozoa or probiotics significantly decreased ($P < 0.001$) the values for potential redox at almost all the points along the curve. The two probiotics tested showed similar effects. The highly significant interactions ($P < 0.001$) between fauna and probiotics (table IV) allowed us to demonstrate that the effect of probiotics was more pronounced in defaunated than in faunated animals (–30 vs –15 mV) (fig 1). The increase of the redox potential values following the meal remained unchanged by

Table II. Effect of fauna and probiotics on the ciliate numbers.

	0	*+SC*	*+AO*	*P*
Total ciliates (10^5/ml)	5.42	7.24	7.16	NS
Entodinium (10^5/ml)	5.31	6.61	6.61	NS
Epidinium (10^4/ml)	0.96	2.86	2.84	NS
Eudiplodinium (10^4/ml)	2.31	3.98	3.39	NS
Isotricha (10^3/ml)	5.73	5.20	5.48	NS

0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*. No differences between means were determined at the level of significance of 5%. P: probiotic effect; NS: nonsignificant.

Table III. Effect of fauna and probiotics on the microflora numbers.

	Fauna		*Probiotic*			*F*	*F(P)*	*F*P*
	D	*F*	*0*	*+SC*	*+AO*			
Total bacterial cells (10^9/ml)	4.60 [a]	3.81 [b]	3.78 [a]	4.36 [b]	4.89 [c]	****	****	NS
Viable bacteria (10^9/ml)	5.27 [a]	1.42 [b]	3.50 [a]	2.60 [a]	3.14 [a]	****	NS	NS
Cellulolytic bacteria (10^8/ml)	3.38 [a]	1.56 [b]	3.29 [a]	1.43 [b]	1.61 [ab]	**	*	NS
Anaerobic fungi (10^3/ml)	4.08 [a]	0.52 [b]	2.42 [a]	2.41 [a]	1.55 [a]	****	NS	NS

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*; [a,b] means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

the presence of the probiotics. However, the inoculation of protozoa into the defaunated rumens greatly decreased the magnitude of the increase observed following feed intake (+ 25 mV in faunated animals vs + 80 mV in defaunated animals).

Rumen pH (table IV) dropped rapidly during the first hours following feeding, reaching a minimum 3 h after the meal and then increasing progressively until the subsequent meal. The statistical analyses revealed that the low pH values observed from 3 h after feeding onwards were significantly increased ($P < 0.05$) by the addition of protozoa to the rumen. AO or SC tended to increase rumen fluid pH throughout the entire period of measurement, although this effect remained significant ($P < 0.05$) only for 1.5 h after the meal. There was no apparent difference between the two probiotics tested on pH. The positive effects of refaunation and probiotics on pH stability were additive.

The ammonia nitrogen (NH_3-N) concentration in the rumen (table IV) increased during the first hour after the meal and then decreased during the following hours, reaching a minimum 5 to 7 h after the meal. The presence of protozoa resulted in a considerable increase in the ruminal NH_3-N concentration ($P < 0.01$), whereas the overall effect of the probiotics was only significant at $P < 0.10$. AO, however, caused a nonsignificant decrease in the NH_3-N concentration in the defaunated animals (fig 1) whereas, in faunated animals, both probiotics increased the NH_3-N concentration, resulting in a significant interaction ($P < 0.05$) between fauna and probiotics. The large differences between the extreme values observed in faunated animals receiving probiotics (175 mg/L) and defaunated animals receiving AO (25 mg/L) deserve attention.

The addition of protozoa in a defaunated rumen increased three-fold the NH_3-N pool (table V). There was a highly significant interaction on the NH_3-N pool between protozoa and probiotics. The increase of the NH_3-N pool in the rumen due to probiotics was higher in refaunated animals in comparison to the defaunated ones.

The proportion of carbon dioxide in the fermentation gases increased to the detriment of that of methane during the first hour following the meal; the proportion of methane then increased regularly (table VI). The proportion of hydrogen remained

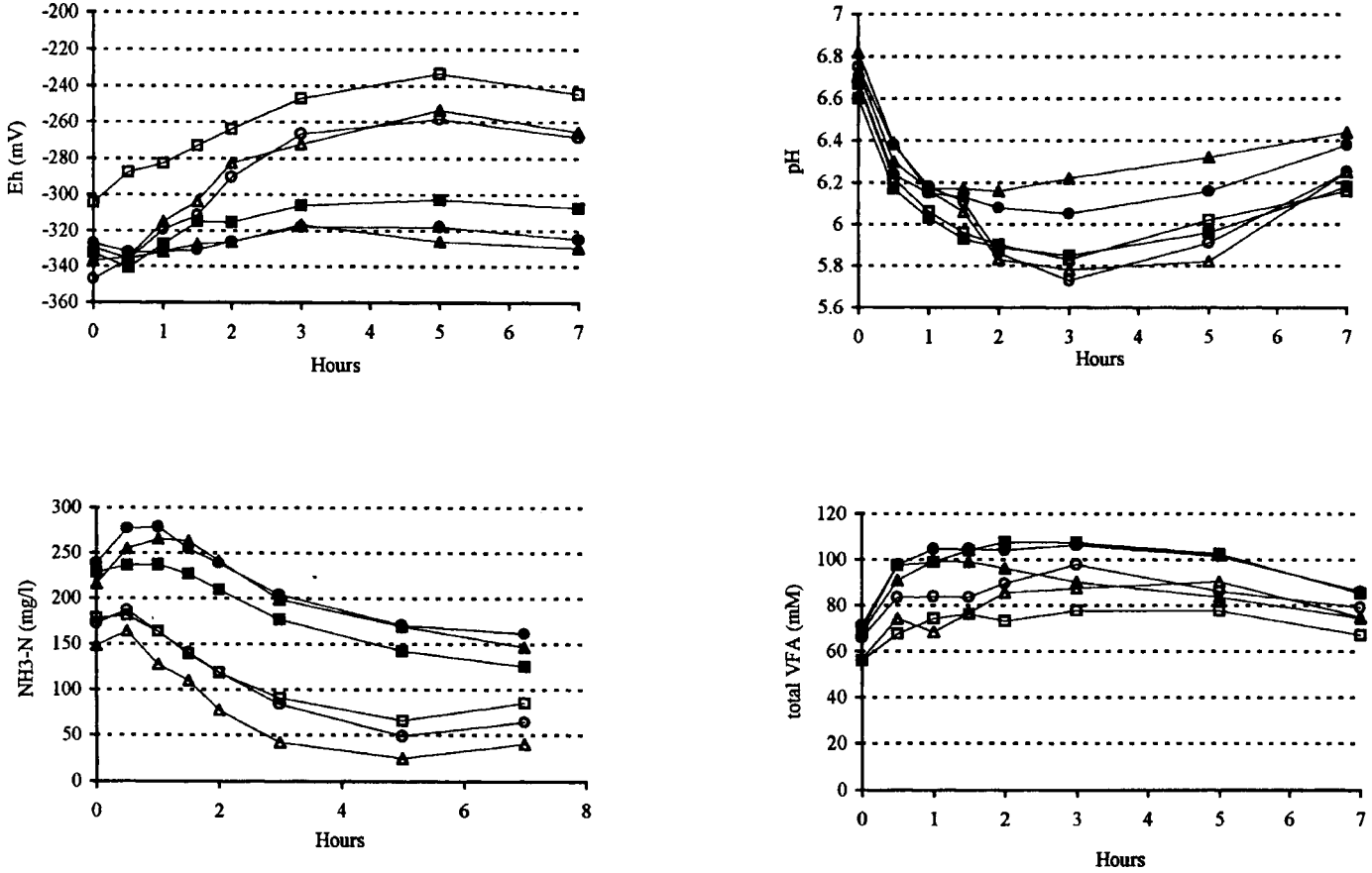

Fig 1. Effect of fauna and probiotics on redox potential, pH, ammonia nitrogen and total volatile fatty acids (VFA). —□— defaunated without probiotic; —▲— defaunated + AO; —●— defaunated + SC; —■— faunated without probiotic; —▲— faunated + AO; —○— faunated + SC;

constantly low (< 1%) between the two meals; it increased during the first hour and decreased constantly during the following hours. The probiotics did not have a significant overall effect on the composition of the fermentation gases. The addition of protozoa to the defaunated rumens caused a significant increase ($P < 0.01$) in the proportion of methane and a decrease in that of carbon dioxide. This effect was particularly visible during the first 3 h following a meal. The proportion of hydrogen in the headspace gas was significantly increased ($P < 0.01$) by the presence of protozoa. There was no interaction between protozoa and probiotics on rumen gases.

Table IV. Effect of fauna and probiotics on redox potential (mV), pH and ammonia nitrogen (NH_3-N, mg/L).

	Time (h) after feeding	*Fauna*		*Probiotic*				
		D	*F*	*0*	*+SC*	*+AO*		
Redox potential								
	0	−323.17 ^{a}	−330.25 ^{b}	−318.42 ^{a}	−337.00 ^{b}	−333.00 ^{b}	F	****
	0.5	−311.42 ^{a}	−337.17 ^{b}	−314.08 ^{a}	−333.83 ^{b}	−335.17 ^{b}	F(P)	****
	1	−300.00 ^{a}	−329.50 ^{b}	−305.08 ^{a}	−325.50 ^{b}	−323.33 ^{b}	F*P	**
	1.5	−290.17 ^{a}	−322.00 ^{b}	−293.92 ^{a}	−321.00 ^{b}	−315.50 ^{b}	T	****
	2	−275.08 ^{a}	−320.75 ^{b}	−289.58 ^{a}	−308.17 ^{b}	−304.33 ^{ab}		
	3	−258.08 ^{a}	−311.67 ^{b}	−276.50 ^{a}	−292.17 ^{a}	−294.33 ^{a}		
	5	−244.58 ^{a}	−312.42 ^{b}	−268.08 ^{a}	−288.17 ^{b}	−289.67 ^{b}		
	7	−255.67 ^{a}	−317.25 ^{b}	−275.92 ^{a}	−296.50 ^{b}	−297.50 ^{b}		
pH								
	0	6.73 ^{a}	6.65 ^{a}	6.63 ^{a}	6.72 ^{ab}	6.77 ^{b}	F	*
	0.5	6.30 ^{a}	6.22 ^{b}	6.19 ^{a}	6.31 ^{b}	6.34 ^{b}	F(P)	**
	1	6.12 ^{a}	6.09 ^{a}	6.05 ^{a}	6.16 ^{b}	6.17 ^{b}	F*P	NS
	1.5	6.02 ^{a}	6.04 ^{a}	5.95 ^{a}	6.12 ^{b}	6.11 ^{b}	T	****
	2	5.87 ^{a}	6.00 ^{a}	5.89 ^{a}	5.97 ^{a}	6.00 ^{a}		
	3	5.79 ^{a}	5.99 ^{b}	5.84 ^{a}	5.89 ^{a}	6.00 ^{a}		
	5	5.94 ^{a}	6.10 ^{b}	5.99 ^{a}	6.03 ^{a}	6.07 ^{a}		
	7	6.21 ^{a}	6.30 ^{b}	6.17 ^{a}	6.32 ^{a}	6.34 ^{a}		
NH_3-N								
	0	169.72 ^{a}	228.00 ^{b}	203.68 ^{ab}	205.70 ^{a}	182.38 ^{b}	F	***
	0.5	178.69 ^{a}	251.22 ^{b}	208.90 ^{a}	232.37 ^{a}	209.63 ^{a}	F(P)	*
	1	154.75 ^{a}	254.47 ^{b}	200.54 ^{ab}	221.06 ^{a}	196.29 ^{b}	F*P	**
	1.5	132.37 ^{a}	242.87 ^{b}	182.89 ^{a}	197.96 ^{a}	186.73 ^{a}	T	****
	2	108.29 ^{a}	224.72 ^{b}	163.90 ^{a}	178.77 ^{a}	159.44 ^{a}		
	3	77.09 ^{a}	189.48 ^{b}	134.00 ^{a}	144.37 ^{a}	120.77 ^{a}		
	5	51.65 ^{a}	156.34 ^{b}	104.42 ^{a}	109.86 ^{a}	97.26 ^{a}		
	7	68.88 ^{a}	139.86 ^{b}	105.37 ^{a}	113.04 ^{a}	93.71 ^{a}		

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*; ^{a,b} means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; T: time effect; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

Table V. Effect of fauna and probiotics on pH and the pools of ammonia nitrogen (NH_3-N) and volatile fatty acids (VFA) in the rumen, 5 h after feeding.

	Fauna		*Probiotic*			*F*	*F(P)*	*F*P*
	D	*F*	*+0*	*+SC*	*+AO*			
pH	5.97 [a]	6.15 [b]	6.09 [a]	6.02 [a]	6.03 [a]	****	NS	NS
NH_3-N (mg)	822.6 [a]	2 575.0 [b]	1 170.9 [a]	2 306.7 [b]	2 146.5 [b]	****	****	****
Total VFA (mmol)	907.5 [a]	1 077.7 [b]	969.2 [a]	1 014.4 [a]	1 017.5 [a]	***	*	*
Acetate (mmol)	594.9 [a]	700.7 [b]	632.9 [a]	648.4 [a]	677.0 [a]	****	**	NS
Propionate (mmol)	168.9 [a]	186.4 [a]	178.3 [a]	184.9 [a]	169.1 [a]	*	NS	NS
Butyrate (mmol)	97.81 [a]	146.28 [b]	116.35 [a]	132.73 [a]	122.76 [a]	****	NS	NS
Valerate (mmol)	14.07 [a]	15.15 [a]	15.12 [a]	14.88 [a]	13.30 [a]	NS	NS	NS
Caproate (mmol)	8.01 [a]	3.39 [b]	5.52 [a]	5.47 [a]	6.27 [a]	****	NS	NS
Isobutyrate (mmol)	9.12 [a]	10.63 [a]	8.92 [a]	11.21 [b]	10.47 [ab]	**	**	**
Isovalerate (mmol)	14.71 [a]	15.13 [a]	12.12 [a]	16.81 [b]	18.61 [b]	NS	***	NS

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*; [a,b] means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

The concentration of total VFAs in the rumen (table VII) increased for 3 h after the meal, then decreased until the following meal (fig 1). The presence of protozoa in the rumen caused a significant increase in the VFA concentrations ($P < 0.05$) for all samples taken. The probiotics had no overall effect on the VFA concentrations. The overall effect of protozoa on the molar proportion of acetate was significant at the threshold of 10%. Only values obtained after the 1 h 30 min period following the meal were significantly depressed by the presence of protozoa (table VII). The molar proportions of propionate, valerate and caproate were significantly reduced, to the advantage of that of butyrate, in the presence of protozoa and at all points of the kinetics studied ($P < 0.05$) (tables VII and VIII). Protozoa caused a drop in the proportion of isobutyrate that was only significant at certain points of the kinetics, whereas they had no global effect on isovalerate.

Probiotics had a significant overall effect ($P < 0.001$) on the molar percentage of acetate which was diminished by the addition of SC and of AO. The significant "fauna x probiotic" interaction ($P < 0.01$), which was outlined on the proportion of acetate, stems from a negative effect of probiotics on this parameter in defaunated animals, whereas no effect was observed in faunated animals. The global effect of probiotics on the molar proportion of valerate, as well as the "fauna x probiotic" interaction, was significant at the 10% threshold. The latter is the result of a negative effect of probiotics in defaunated animals whereas the treatment had no effect in refaunated animals. Lactate was only found in traces, or not at all, in rumen samples and was therefore disregarded in this work.

The pool of total VFAs in the rumen digesta obtained from the rumen emptying was not significantly altered by probiotics (table V). SC tended to decrease the total VFAs in the absence of protozoa and to

Table VI. Effect of fauna and probiotics on rumen gases (%).

	Time (h) after feeding	Fauna		Probiotic				
		D	F	0	+SC	+AO		
CO_2								
	0	60.38 [a]	57.76 [a]	58.01 [a]	60.87 [a]	59.40 [a]	F	***
	1	74.96 [a]	72.98 [b]	74.81 [a]	73.35 [a]	72.90 [a]	F(P)	NS
	2	72.54 [a]	70.28 [b]	72.31 [a]	70.65 [a]	70.35 [a]	F*P	NS
	3	71.87 [a]	69.51 [b]	71.78 [a]	69.89 [ab]	69.28 [b]	T	****
	7	67.97 [a]	66.33 [a]	68.20 [a]	65.85 [a]	66.33 [a]		
CH_4								
	0	39.54 [a]	42.24 [a]	41.92 [a]	39.22 [a]	40.52 [a]	F	***
	1	24.76 [a]	26.36 [a]	24.54 [a]	26.58 [a]	26.58 [a]	F(P)	NS
	2	27.32 [a]	29.58 [b]	27.54 [a]	29.12 [a]	29.58 [a]	F*P	NS
	3	27.99 [a]	30.34 [b]	28.06 [a]	30.03 [b]	30.49 [b]	T	****
	7	31.96 [a]	33.60 [a]	31.72 [a]	34.07 [a]	33.60 [a]		
H_2								
	0	0.0045 [a]	0.0225 [b]	0.0069 [a]	0.0279 [a]	0.0100 [a]	F	****
	1	0.2578 [a]	0.6660 [b]	0.5132 [a]	0.4002 [ab]	0.3395 [b]	F(P)	NS
	2	0.1406 [a]	0.1738 [a]	0.1664 [a]	0.1599 [a]	0.1346 [a]	F*P	NS
	3	0.0948 [a]	0.1466 [a]	0.1169 [a]	0.1346 [a]	0.1108 [a]	T	****
	7	0.0306 [a]	0.0713 [b]	0.0625 [a]	0.0335 [a]	0.0400 [a]		

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*; [a,b] means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; T: time effect; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

increase them in faunated animals. The total VFAs had a tendancy to decrease with AO in defaunated rumens whereas no change occurred in faunated rumens. This explains the presence of a "fauna x probiotic" interaction ($P < 0.10$). A significant increase in the branched VFA pools in the rumen was observed after the animals being treated with probiotics. The pool of lactate was negligible. The protozoa significantly increased the pool of total VFAs ($P < 0.01$), acetate ($P < 0.001$) and butyrate ($P < 0.001$). Furthermore, the pool of propionate was slightly increased ($P < 0.10$) whereas the pool of caproate decreased more than two-fold ($P < 0.001$) in faunated animals. The significant ($P < 0.05$) interaction between fauna and probiotics on isobutyrate result from a positive effect of SC only noted in refaunated animals while AO increased this parameter mainly in defaunated animals.

DISCUSSION

The increase in redox potential after the meal is mainly due to the supply of oxygen directed towards the rumen during feed intake, mastication and water intake. Due to stimulation of the blood flow in the rumen mucosa during feeding (Barnes et al, 1983), more oxygen may also be exchanged with

Table VII. Effect of fauna and probiotics on total volatile fatty acids (mM) and molar percentage of acetate (C_2), propionate (C_3) and butyrate (C_4).

	Time (h) after feeding	*Fauna*		*Probiotic*				
		D	*F*	*0*	*+SC*	*+AO*		
Total VFA								
	0	58.68 ^{a}	69.54 ^{b}	62.67 ^{a}	68.65 ^{a}	62.45 ^{a}	F	****
	0.5	73.24 ^{a}	90.86 ^{b}	77.56 ^{a}	90.44 ^{b}	82.62 ^{ab}	F(P)	NS
	1	75.24 ^{a}	100.45 ^{b}	86.67 ^{ab}	94.16 ^{a}	83.90 ^{b}	F*P	NS
	1.5	78.24 ^{a}	102.94 ^{b}	90.16 ^{a}	94.12 ^{a}	87.92 ^{a}	T	****
	2	80.39 ^{a}	103.80 ^{b}	90.37 ^{a}	96.82 ^{a}	90.80 ^{a}		
	3	85.18 ^{a}	103.03 ^{b}	92.64 ^{ab}	102.05 ^{a}	89.08 ^{b}		
	5	83.03 ^{a}	97.45 ^{b}	90.04 ^{a}	93.80 ^{a}	87.06 ^{a}		
	7	72.10 ^{a}	82.89 ^{b}	76.24 ^{a}	82.63 ^{a}	74.88 ^{a}		
C_2								
	0	67.34 ^{a}	65.56 ^{a}	68.42 ^{a}	64.05 ^{b}	64.85 ^{b}	F	*
	0.5	65.54 ^{a}	63.36 ^{a}	65.94 ^{a}	62.95 ^{a}	62.95 ^{a}	F(P)	****
	1	63.78 ^{a}	62.15 ^{a}	64.11 ^{a}	61.55 ^{a}	62.08 ^{a}	F*P	****
	1.5	62.79 ^{a}	62.46 ^{a}	63.80 ^{a}	61.36 ^{a}	61.53 ^{a}	T	****
	2	67.66 ^{a}	63.02 ^{b}	68.83 ^{a}	61.72 ^{b}	61.90 ^{b}		
	3	67.78 ^{a}	64.37 ^{b}	69.16 ^{a}	62.76 ^{b}	63.11 ^{b}		
	5	66.98 ^{a}	65.51 ^{a}	68.29 ^{a}	63.88 ^{a}	64.46 ^{a}		
	7	71.83 ^{a}	66.23 ^{b}	73.05 ^{a}	64.59 ^{b}	65.28 ^{b}		
C_3								
	0	21.61 ^{a}	16.69 ^{b}	19.93 ^{a}	18.79 ^{ab}	17.76 ^{b}	F	****
	0.5	23.16 ^{a}	20.29 ^{b}	22.11 ^{a}	21.72 ^{a}	20.91 ^{a}	F(P)	NS
	1	23.49 ^{a}	21.05 ^{b}	22.28 ^{a}	22.92 ^{a}	21.56 ^{a}	F*P	NS
	1.5	23.61 ^{a}	20.92 ^{b}	22.33 ^{a}	22.79 ^{a}	21.54 ^{a}	T	****
	2	23.89 ^{a}	20.42 ^{b}	22.88 ^{a}	22.22 ^{a}	20.58 ^{b}		
	3	22.46 ^{a}	19.45 ^{b}	21.65 ^{a}	21.07 ^{a}	19.41 ^{a}		
	5	20.09 ^{a}	18.56 ^{b}	20.02 ^{a}	19.68 ^{a}	17.61 ^{b}		
	7	21.09 ^{a}	17.74 ^{b}	20.70 ^{a}	19.04 ^{ab}	17.19 ^{b}		
C_4								
	0	8.85 ^{a}	13.00 ^{b}	10.59 ^{a}	11.10 ^{a}	11.05 ^{a}	F	***
	0.5	10.24 ^{a}	12.80 ^{b}	11.65 ^{a}	10.98 ^{a}	11.68 ^{a}	F(P)	NS
	1	10.85 ^{a}	13.08 ^{b}	12.22 ^{a}	11.36 ^{a}	11.98 ^{a}	F*P	NS
	1.5	11.26 ^{a}	13.33 ^{b}	12.44 ^{a}	11.64 ^{a}	12.57 ^{a}	T	****
	2	12.05 ^{a}	13.22 ^{a}	12.25 ^{a}	11.89 ^{a}	12.95 ^{a}		
	3	12.33 ^{a}	13.15 ^{a}	12.92 ^{a}	12.03 ^{a}	13.10 ^{a}		
	5	11.86 ^{a}	13.03 ^{a}	12.16 ^{a}	12.22 ^{a}	13.23 ^{a}		
	7	12.17 ^{a}	13.09 ^{a}	12.78 ^{a}	12.17 ^{a}	12.78 ^{a}		

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae*; VFA: volatile fatty acids; ^{ab} means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; T: time effect; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

Table VIII. Effect of fauna and probiotics on molar percentage of valerate (C_5), caproate (C_6), isobutyrate (IC_4) and isovalerate (IC_5).

	Time (h) after feeding	*Fauna*		*Probiotic*				
		D	*F*	*0*	*+SC*	*+AO*		
C_5								
	0	1.24 [a]	1.03 [b]	1.16 [a]	1.16 [a]	1.06 [a]	F	***
	0.5	1.29 [a]	1.08 [b]	1.24 [a]	1.11 [a]	1.13 [a]	F(P)	*
	1	1.47 [a]	1.25 [b]	1.41 [a]	1.29 [a]	1.32 [a]	F*P	*
	1.5	1.55 [a]	1.38 [b]	1.49 [a]	1.43 [a]	1.45 [a]	T	****
	2	1.75 [a]	1.45 [b]	1.63 [a]	1.53 [a]	1.59 [a]		
	3	1.77 [a]	1.45 [b]	1.66 [a]	1.55 [a]	1.54 [a]		
	5	1.67 [a]	1.30 [b]	1.49 [a]	1.46 [a]	1.47 [a]		
	7	1.69 [a]	1.12 [b]	1.51 [a]	1.27 [a]	1.27 [a]		
C_6								
	0	0.39 [a]	0.22 [b]	0.29 [a]	0.24 [a]	0.38 [a]	F	****
	0.5	0.36 [a]	0.19 [b]	0.25 [a]	0.21 [a]	0.36 [a]	F(P)	NS
	1	0.42 [a]	0.16 [b]	0.02 [a]	0.02 [a]	0.02 [a]	F*P	NS
	1.5	0.46 [a]	0.16 [b]	0.30 [a]	0.25 [a]	0.32 [a]	T	****
	2	0.71 [a]	0.17 [b]	0.45 [a]	0.28 [a]	0.41 [a]		
	3	0.72 [a]	0.17 [b]	0.44 [a]	0.32 [a]	0.40 [a]		
	5	0.82 [a]	0.18 [b]	0.45 [a]	0.37 [a]	0.50 [a]		
	7	0.81 [a]	0.17 [b]	0.48 [a]	0.32 [a]	0.45 [a]		
IC_4								
	0	2.04 [a]	1.69 [b]	1.87 [a]	1.80 [a]	1.90 [a]	F	**
	0.5	1.31 [a]	1.14 [b]	1.28 [a]	1.18 [a]	1.16 [a]	F(P)	NS
	1	1.15 [a]	1.04 [b]	1.14 [a]	1.03 [a]	1.08 [a]	F*P	NS
	1.5	1.05 [a]	0.99 [a]	1.04 [a]	0.99 [a]	1.00 [a]	T	****
	2	1.01 [a]	0.95 [a]	1.00 [a]	0.92 [a]	0.99 [a]		
	3	0.92 [a]	0.87 [a]	0.90 [a]	0.86 [a]	0.92 [a]		
	5	0.98 [a]	0.84 [a]	0.89 [a]	0.87 [a]	0.97 [a]		
	7	1.19 [a]	0.88 [b]	1.01 [a]	1.00 [a]	1.11 [a]		
IC_5								
	0	2.64 [a]	2.49 [a]	2.50 [a]	2.64 [a]	2.63 [a]	F	NS
	0.5	1.62 [a]	1.71 [a]	1.63 [a]	1.73 [a]	1.67 [a]	F(P)	NS
	1	1.37 [a]	1.58 [b]	1.42 [a]	1.51 [a]	1.55 [a]	F*P	NS
	1.5	1.21 [a]	1.49 [b]	1.27 [a]	1.42 [a]	1.43 [a]	T	****
	2	1.26 [a]	1.39 [a]	1.27 [a]	1.34 [a]	1.42 [a]		
	3	1.15 [a]	1.30 [a]	1.12 [a]	1.30 [a]	1.37 [a]		
	5	1.34 [a]	1.30 [a]	1.18 [a]	1.39 [ab]	1.56 [b]		
	7	1.64 [a]	1.33 [b]	1.37 [a]	1.49 [a]	1.70 [a]		

D: defaunated animals; F: faunated animals; 0: without probiotic; +SC: with *Saccharomyces cerevisiae*; +AO: with *Aspergillus oryzae;* VFA: volatile fatty acids; [ab] means with different letters are significantly different ($P < 0.05$); F: fauna effect; F(P): effect of probiotic within fauna; F*P: interaction between fauna and probiotic; T: time effect; NS: nonsignificant; * significant ($P < 0.10$); ** significant ($P < 0.05$); *** significant ($P < 0.01$); **** significant ($P < 0.001$).

the blood compartment through the rumen wall throughout this period. The decrease in redox potential in response to the introduction of probiotics is in agreement with the results of Newbold et al (1993) who revealed, through the use of the Rusitec in vitro system, that yeasts consume oxygen during respiration and that, consequently, a lowered oxygen concentration stimulates an increase of the rumen anaerobic bacteria populations. This result could demonstrate the necessity of introducing, in the rumen, probiotics in their live or metabolically active form. As these aerobic organisms do not multiply within the rumen and because they are continuously eliminated, as observed by Kumar et al (1994), it is unlikely that each daily dose would consume enough oxygen to modify the redox potential for as long as 24 h. A stimulation of the facultative anaerobes and the subsequent consumption of oxygen, possibly due to the production of unidentified growth factors from probiotics, could also occur in the presence of probiotics.

Hydrogen production and oxygen consumption by the protozoa in their cytosol or in hydrogenosomes (Yarlett et al, 1983), confirmed by the analyses which we carried out on the rumen gases, were probably responsible for the decrease in redox potential observed following refaunation.

The drop in pH after feeding confirmed those already observed with high starch diets, the latter representing 26% of the dry matter in the diet used in this experiment. The pH measured before feeding was already lower than that measured on forage-based diets.

According to the literature, the in vivo effect of probiotics on the pH values of the rumen content is variable. In agreement with our results, Newbold et al (1990) and Kumar et al (1994), noted that the probiotics stabilized the pH while other authors did not observe any effect of the probiotics (Martin and Nisbet, 1990; Caton et al, 1993). In fact, some even observed a decrease in pH (Harrison et al, 1988; Edwards et al, 1990). The buffering effect of the probiotics could result in part from a decrease in the lactate concentrations in the rumen (Edwards, 1991; Newbold et al, 1992). Indeed, Nisbet and Martin (1990 and 1991) demonstrated that SC and AO stimulate the uptake of lactate by *Selenomonas ruminantium*. Waldrip and Martin (1993) revealed the positive effect of AO on lactate consumption by *Megasphaera elsdenii*. However, in our study, the lactate concentration remained always under the limit of detection (≤ 1 mM); thus, no clear effect of the probiotics on this parameter could be revealed. As the animals were adapted progressively to the high barley diet for 1 month before starting the experiment, we can suggest that the microflora was not disturbed by these extreme conditions leading to a very low concentration of lactate in the rumen as indicated by Nakamura et al (1989).

The absence of an effect by the ciliate protozoa on the rumen pH in the animals not receiving probiotics (fig 1) is in agreement with some authors (De Smet et al, 1992; Jouany et al, 1995), although Ivan et al (1991) and Nagaraja et al (1992) revealed an increase in pH in faunated animals whereas, in other studies, a decrease in pH was observed (Jouany and Sénaud, 1982, 1983; Broudiscou et al, 1994). Our results clearly demonstrated that the addition of probiotics to a defaunated sheep had no lasting effect on the ruminal pH values. On the contrary, pH increased by 0.2 and 0.3 units 3 h after feeding when SC and AO were added respectively to the refaunated rumens (fig 1). This could indicate that protozoa are involved in the effect of probiotics on the increase of rumen pH while bacteria are not.

Mould et al (1983/1984) suggested that pH 6 is a limiting value for the correct functioning of the rumen and that the duration for which the pH remains below this value

can be regarded as a detrimental indicator of cellulolysis. In the current study, only the simultaneous presence of protozoa and probiotics allowed the constant maintenance of such favorable conditions in the rumen. Surprisingly, these conditions had no stimulating effect on the growth of cellulolytic bacteria (table III). In the absence of the protozoa, the rumen pH remained below 6 for 5 of the 7 h studied, even in the presence of probiotics, and the highest numbers of cellulolytic bacteria were obtained in that condition.

Most of the literature data indicate that SC or AO have no effect on ammonia nitrogen concentration in the rumen (Frumholtz, 1991; Varel and Kreikemeier, 1994a, b). Nevertheless, in a more limited number of studies, a drop in ammonia nitrogen was observed in animals receiving SC (Newbold et al, 1990; Frumholtz, 1991). Beharka et al (1991) noted a tendancy towards an increase in ammonia nitrogen concentration only during the first weeks of the addition of AO. Since the ammonia pool within the rumen is a result of the difference between its formation by deamination of amino acids and urea on the one hand, and its elimination through the synthesis of microbial proteins and the outflow of rumen liquid on the other, its evolution will depend on the effect of probiotics on these parameters. Some studies have shown that probiotics stimulate the flow of microbial proteins in the small intestine and, consequently, increase bacterial ammonia nitrogen fixation (Edwards et al, 1990; Erasmus et al, 1992). The increase in ruminal NH_3-N concentration, which we observed only with refaunated animals in our experiment after the addition of SC or AO (fig 1), indicates that protozoa interact with the response of the probiotics tested. As confirmed by the higher isovalerate concentration in the refaunated probiotic-treated animals, this evolution was mainly explained by a higher production of ammonia from the degradation and fermentation of proteins or peptides in the rumen. As a possible hypothesis, it could be that some oligopeptides are released by probiotics in the rumen and are then used by the protozoa to produce smaller peptides or amino acids that are finally deaminated by other protozoa and bacteria (Jouany, 1996). Probiotics can also stimulate the ciliate growth and their activity against proteins in the rumen. Indeed, in contrast to Kumar et al (1994), the number of protozoa was not significantly stimulated by probiotics. An increase in the return of urea through the rumen wall could also be considered as a possible hypothesis but there is no explanation for this phenomenon.

The marked increase in rumen ammonia nitrogen concentration in all samples taken after the addition of protozoa into the defaunated rumens was observed in all former studies (Broudiscou et al, 1994; Jouany et al, 1995), and is a result of the consumption of bacterial and food proteins by the protozoa which cannot carry out de novo synthesis of amino acids from the simple forms of nitrogen-like ammonia (Williams and Coleman, 1992).

The effect of probiotics on methane production is unclear. The absence of an effect observed in the current study does not coincide with the increased methanogenesis which was revealed in vitro by Martin et al (1989) with SC. This in turn conflicts with the decrease noted by Frumholtz et al (1989) with AO in Rusitec and by Williams (1988) with SC in an in vivo experiment. Nisbet and Martin (1990), also in vitro, demonstrated that the addition of 0.7 g/L of AO to rumen simulators containing hay and an inoculum which was nonadapted to the probiotic, decreased methane production; this effect disappeared when fermentations were performed with an inoculum which was adapted to the probiotic. It would appear, therefore, that the evolution of this parameter might depend on the conditions in the rumen environment that are mainly gover-

ned by the resident microbial population and the type of diet.

The increase in the proportion of methane in the fermentation gases in animals whose rumen was reinoculated with protozoa was in agreement with the results obtained in vivo by Whitelaw et al (1984) and Jouany et al (1995). This result is due to protozoal hydrogen production as discussed earlier, and its utilization by methanogenic bacteria present in the immediate vicinity or fixed to the outside pellicle of ciliates. This symbiosis is, by definition, favorable to the metabolism of both types of organisms. The metabolic pathways thus employed for hydrogen utilization can explain the limited effect of protozoa on the production of propionate.

In agreement with our results, the literature reveals that SC does not have an obvious effect on total VFA concentrations (Newbold et al, 1990) or it has a slightly positive effect (Frumholtz, 1991; Kumar et al, 1994) confirmed by in vitro measurements of VFA productions (Gray and Ryan, 1989; Ryan and Gray, 1989). AO did not stimulate the VFA concentrations either (Frumholtz, 1991; Varel and Kreikemeier, 1994a). The effect of probiotics on the composition of the mixture of VFAs was equally difficult to characterize. We confirmed the tendancy noted by other authors of a slight increase in the acetate/propionate ratio with AO (Frumholtz et al, 1989; Newbold et al, 1990); however, these effects are generally nonsignificant (Wallace and Newbold, 1992).

The increase in the concentration of total VFAs, linked to the presence of protozoa, confirms the results of Jouany and Sénaud (1982), and Broudiscou et al (1994), whereas Frumholtz (1991) and Jouany et al (1995) did not observe any significant effect of protozoa on the concentration of total VFAs. Like most authors, we noted that the presence of the protozoa increased the molar proportion of butyrate in the rumen (Jouany et al, 1988). This increase usually occurs either to the detriment of acetate (Demeyer et al, 1982; Frumholtz, 1991), or to that of propionate (Itabashi et al, 1982; Jouany and Sénaud, 1982).

This study demonstrated that the two probiotics tested, SC (I-1096) and AO, created new conditions in the rumen by decreasing the redox potential while limiting the drop in pH which occurs in animals after feeding a diet rich in highly fermentable starch. Among the significant interactions between the protozoa and probiotics, was the fact that the effects of both treatments on the redox potential, and to a lesser degree the pH, were accumulative.

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