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Microbial fermentation of a high forage diet in sheep rumen, semi-continuous (Rusitec) and continuous culture systems

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SUMMARY – The aim of this study was to compare the rumen fermentation in two types of fermenters [continuous (CF) and semi-continuous (Rusitec)] inoculated with rumen content from sheep to that in the rumen of sheep fed a high forage diet. Whereas in both in vitro systems pH values remained fairly stable through the day (pH>6.5), rumen pH dropped markedly after feeding, and values at 2, 4 and 6 h sampling were lower (P<0.01) than those observed in fermenters. Concentration of total volatile fatty acids was greatest (P<0.05) in vivo and lowest (P<0.05) in Rusitec, with CF having an intermediate value. There were no differences (P>0.05) in the acetate:propionate ratio found in the rumen and in Rusitec, but CF showed a greater (P<0.05) value. There were no differences (P>0.05) between fermenters in the diet apparent disappearance from nylon bags after 48 h of incubation, but this value was greater (P<0.05) in rumen. Efficiency of microbial growth in sheep was lower (P<0.05) than that in CF, but greater (P<0.05) than in Rusitec (22.1, 35.1 and 12.8 mg microbial N/g OM apparently fermented in the rumen, respectively).

Keywords: Rumen fermentation, sheep, rusitec, continuous fermenter.

RESUME – *"Fermentation microbienne d'un régime à base de fourrage dans le rumen de mouton, et dans un système de culture semi-continu (Rusitec) et continu". On a comparé la fermentation ruminale dans deux types de fermenteurs (continu -CF- et semi-continu -Rusitec-) inoculés avec du jus du rumen de brebis, à celle obtenue dans le rumen de brebis qui ont reçu une ration riche en fourrages. Tandis que dans les deux systèmes in vitro les valeurs du pH sont restées assez stables durant le jour (pH>6,5), le pH du rumen a chuté nettement après l'ingestion d'aliment, et les valeurs des prélèvements de 2, 4 et 6 h ont été inférieures (P<0,01) à celles observées dans les deux types de fermenteurs. La concentration en acides gras volatils totaux in vivo était la plus élevée (P<0,05) tandis que celle dans le Rusitec était la plus faible (P<0,05) et celle du système CF montrait des valeurs intermédiaires. Il n'y a eu aucune différence (P>0,05), concernant le rapport acide acétique:acide propionique, entre les valeurs du rumen de brebis et celles du Rusitec ; mais la valeur des CF était plus élevée (P<0,05). La disparition de l'aliment des sacs de nylon après 48 h d'incubation n'est pas différente (P>0,05) quand on compare les deux systèmes de fermenteurs mais cette valeur était plus élevée (P<0,05) dans le rumen des brebis. Le rendement de la protéosynthèse chez les brebis a été inférieur (P<0,05) à celui des CF, mais plus grand (P<0,051) que dans le Rusitec (22,1 ; 35,1 et 12,8 mg azote microbien/g matière organique apparemment fermentée dans le rumen, respectivement).*

Mots-clés : *Fermentation ruminale, ovins, Rusitec, fermenteurs à flux continu.*

Introduction

Most research on rumen fermentation has been carried out with fistulated animals, although these studies are expensive and laborious. Moreover, with the rumen being a very complex system, it is difficult to study its function *in vivo*. These problems, together with the increased public awareness of the animal rights, and as a consequence, the need for decreasing the number of fistulated animals used for experimental purposes have contributed to the development of *in vitro* technologies for simulating rumen fermentation. Many types of artificial rumen apparatus have been described in the literature, but two of the most widely used in Europe are the *Rusitec*, designed by Czerkawski and Breckenridge (1977) and the continuous fermenters (*CF*), such as those designed by Miettinen and Setälä (1989). Although these *in vitro* systems are used in many laboratories, direct comparisons between *in vivo* and *in vitro* rumen fermentation are limited (Hannah *et al.*, 1986; Mansfield *et al.*, 1995), and no comparative studies between continuous and Rusitec fermenters have been found in the literature. The objective of this study was to compare the rumen fermentation and microbial protein synthesis in sheep, *Rusitec* and *CF*, the working conditions and management of both types of fermenters being those generally used in each of the participant laboratories.

Materials and methods

A complete diet, composed of chopped alfalfa hay (80%; fresh matter basis) and concentrate (20%), was used in all experiments. Concentrate was based on cracked barley grains, cracked corn grains and soy-bean meal (39:44:17 g/100 g; on a fresh matter basis). Sugar beet molasses and a mineral-vitamin mixture was added to the diet at rates of 3.5 and 3.0 g per 100 g of dry matter (DM), respectively. Organic matter, neutral-detergent fibre (NDF) and crude protein contents of diet were 867, 332 and 166 g/kg DM, respectively.

In vivo trial

Four rumen-fistulated Merino sheep were given 1.06 kg DM of diet daily, offered in two equal portions at 9.00 and 21.00 h. The experiment included an adaptation period of 21 days. On day 22 and just before feeding, 2 g of Co-EDTA were injected into the rumen of each sheep to estimate the rumen liquid outflow rate. Rumen contents were sampled just before marker administration and 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h after dosing. Samples of about 50 g were obtained through the cannula and filtered through four layers of cheesecloth. The pH of the fluid was measured immediately and sub-samples were taken for volatile fatty acids (VFA), ammonia-N and Co analyses. On day 25, two nylon bags (46 μm pore size) containing 5 g of the diet were incubated in the rumen of each sheep for 48 h. On day 28, animals were moved to metabolic cages and total collection of faeces and urine was made for 6 days. The urinary excretion of purine derivatives was used to estimate the microbial N flow to the duodenum. More details about the *in vivo* trial are given in Carro *et al.* (2000).

Semi-continuous fermenters (*Rusitec*) trial

One 13-d incubation trial was carried out using a *Rusitec* unit, consisting of four vessels with an effective volume of 600 ml each. The general incubation procedure was as described by Czerkawski and Breckenridge (1977). Fermenters were inoculated with 400 ml of rumen liquor and 80 g of solid rumen content from sheep fed alfalfa hay at maintenance level. Each vessel received daily 15.6 g DM of the diet fed into nylon bags (100 μm pore size) at 9:00 h. A continuous infusion of artificial saliva (McDougall, 1948; pH = 8.4), at a rate of 600 ml/day, was maintained into the vessels. A solution of $SO_4(^{15}NH_4)_2$ was added to the artificial saliva in order to determine microbial growth. On days 9, 10, and 11 the pH of vessel fluid was determined immediately before feeding, and samples of the liquid effluent were collected for VFA and ammonia-N determination. One nylon bag from each vessel was collected daily, washed twice with 40 ml artificial saliva and then washed in the cold rinse cycle of a washing machine. The DM apparent disappearance, after 48 h of incubation, was calculated from the weight loss after oven drying at 60ºC for 48 h, and the residues were analysed for ash. During days 12 and 13 microbial protein synthesis was determined as described by Carro and Miller (1999).

Continuous fermenter trial

One 14-d incubation trial was carried out using four continuous fermenters (Miettinen and Setälä, 1989) with an effective volume of 1100 ml each. The general incubation procedure was as described by Martín García (2001). Fermenters were inoculated with 700 ml of rumen liquor from sheep fed alfalfa hay at maintenace level. Each fermenter received daily 27 g DM of diet administered in two equal portions at 8:00 and 16:00 h. Artificial saliva (McDougall, 1948) was infused continuously into the fermenters at a rate of 900 ml/d and ^{15}N [$SO_4(^{15}NH_4)_2$] was used as a microbial marker. On days 9, 10, and 11, the pH in the fermenters was determined immediately before the morning feeding and samples of the effluents were collected for VFA and ammonia-N determination. On day 12, two nylon bags (46 μm pore size) containing 1.3 g diet were incubated in each fermenter for 48 h. During days 13 and 14 microbial protein synthesis was determined following the same procedure used in the *Rusitec* trial.

Analytical procedures and statistical analyses

Procedures for determination of DM, ash, NDF, N, VFA and ammonia-N, and preparation of samples for ^{15}N analysis of digesta and bacterial pellets have been reported by Carro and Miller (1999). Analytical procedures used in the *in vivo* trial have been described by Carro *et al.* (2000).

The effect of the culture (sheep, *Rusitec* and *CF*) on the main fermentation parameters was tested by ANOVA. When a significant F value ($P<0.05$) was detected, means were compared using the least significant difference test.

Results and discussion

Values for the main fermentation parameters in sheep rumen, *CF* and *Rusitec* are presented in Table 1. As it has been pointed out by Mansfield *et al.* (1995), only data with similar units, i.e. proportions and concentrations, could be statistically compared because absolute amounts of input and outflow of fermenters and sheep differed in numerical magnitude. In general, coefficients of variation of the measured parameters were greater for sheep than for both types of fermenters: lower than 6% on average for both *in vitro* systems and from 2 to 12 % for sheep.

Table 1. Mean values of liquid dilution rate (h^{-1}), pH, concentration of volatile fatty acids (VFA; mmol/l), molar proportions (mol/mol) of individual VFA, concentration of ammonia-N (mg/l), dry matter apparent disappearance from nylon bags after 48 h incubation (DMD; %), microbial protein synthesis (mg microbial N/d) and efficiency of microbial growth (EMG) in sheep rumen, continuous fermenters and *Rusitec* fed a 80:20 alfalfa hay:concentrate diet

Item	Sheep rumen	Continuous fermenters	*Rusitec*	SED
Liquid dilution rate	0.0467^{b}	0.0329^{a}	0.0446^{b}	0.00184
Average pH†	6.50^{a}	6.64^{b}	6.91^{c}	0.062
pH before feeding	6.98^{b}	6.69^{a}	7.03^{b}	0.066
Total VFA††	132^{c}	121^{b}	84.2^{a}	2.87
Molar proportions of:				
Acetate	65.5^{b}	68.7^{c}	57.0^{a}	0.81
Propionate	17.2^{b}	14.3^{a}	16.7^{b}	0.79
Butyrate	13.5^{b}	12.2^{a}	16.2^{c}	0.48
Isobutyrate	1.09^{c}	1.01^{b}	0.93^{a}	0.030
Isovalerate	1.41^{a}	1.72^{b}	3.58^{c}	0.053
Valerate	1.27^{a}	1.98^{b}	5.53^{c}	0.054
Acetate:Propionate	3.84^{a}	4.80^{b}	3.40^{a}	0.214
NH_3-N††	218^{b}	171^{a}	251^{c}	9.6
DMD	75.9^{b}	59.7^{a}	62.7^{a}	1.51
Microbial N	-	698^{b}	112^{a}	30.8
EMG†††	22.1^{b}	35.1^{c}	12.8^{a}	3.09

†Values averaged over 12 h sampling period.
††Values averaged over 24 h sampling period for sheep rumen and effluents.
†††EMG: mg microbial N/g organic matter apparently digested in the rumen. Organic matter apparently digested in the rumen of sheep was estimated as 85% of total tract organic matter digestibility.
$^{a, b, c}$Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).

There were no differences ($P>0.05$) between sheep and *Rusitec* in the fractional outflow rate of the liquid phase, but *CF* presented a lower ($P<0.05$) value. Concentration of total VFA was highest ($P<0.05$) in sheep rumen and lowest ($P<0.05$) in *Rusitec* (132 and 84.2 mmol/l, respectively), with *CF*

having an intermediate value (121 mmol/l). Whereas there were no differences between sheep rumen and *Rusitec* in the proportion of propionate (17.2 and 16.7%, respectively), *CF* showed a lower value (P<0.05; 14.3%). *CF* showed the lowest (P<0.05) propionate and butyrate molar proportions, but the greatest (P<0.05) acetate proportion. Consequently, acetate to propionate ratios *in vivo* and in *Rusitec* (3.84 and 3.40, respectively) were lower (P<0.05) than in *CF* (4.80). These shifts in proportions of major VFA between *in vivo*, *CF* and *Rusitec* could be due to differences in pH, dilution rate and solids retention time, all of which can alter VFA proportions (Russell, 1998; Meng *et al.*, 1999). Moreover, differences between *in vivo* and *in vitro* concerning 'diet:volume' ratio should be taken into account. That ratio was much higher for sheep (about 152 for a mean rumen volume of 7 l) than for both types of fermenters (26 and 25 for *Rusitec* and *CF*, respectively).

There were no differences between sheep rumen and *Rusitec* in the pH before feeding (6.98 and 7.03, respectively), but the value in *CF* was lower (P<0.05; 6.69). However, pH evolution after feeding differed markedly among fermentation systems. Whereas in both types of fermenters pH values remained fairly stable through the day (values were always higher than 6.5), rumen pH in sheep dropped markedly after feeding (see Fig. 1), and values at 2, 4 and 6 h sampling were lower (P<0.01) than those observed in both *in vitro* systems. The high pH values observed in *Rusitec* and *CF* are due to the high buffer capacity of the artificial saliva used in both *in vitro* systems and to the lower amounts of VFA produced compared to *in vivo*.

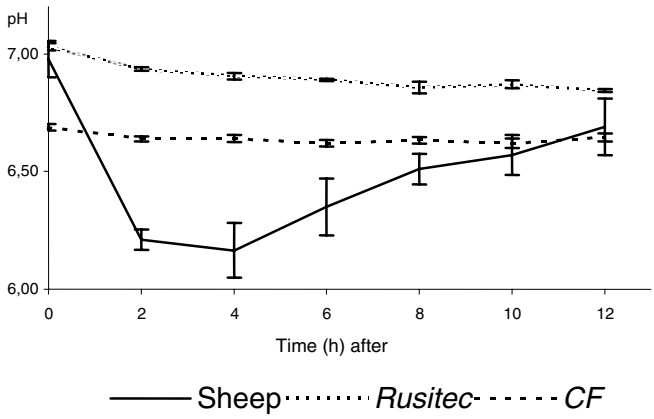

Fig. 1. Evolution of pH after feeding in sheep rumen, *Rusitec* and continuous fermenters (*CF*).

DM disappearance of diet from nylon bags (DMD) incubated in sheep rumen was greater (P<0.05; 75.9%) than that observed in both *in vitro* systems, probably due to the fact that microbial concentrations in fermenters are much lower than in the *in vivo* rumen. Microbial concentrations in *CF* are greater than those in *Rusitec* (see daily microbial N production in Table 1) and therefore, a greater DMD from nylon bags in *CF* should be expected. However, there were no differences (P>0.05) between *Rusitec* and *CF* in the DMD of diet from nylon bags incubated for 48 h (62.7 and 59.7%, respectively). This lack of differences between both types of fermenters could be due to a compensation of expected differences ascribed to the microbial concentrations in both systems by the different pore size of the nylon bags used for the incubations (46 μm and 100 μm in *CF* and in *Rusitec*, respectively). In fact, Carro *et al.* (1995) studied the effects of bag pore size in *Rusitec* system when a high-forage diet was incubated, and reported that the use of 100 μm bags increased the DM disappearance by 9.5% compared to those of 40 μm.

As expected, daily microbial N production was greater in *CF* than in *Rusitec* (P<0.05; 698 and 112 mg microbial N/d, respectively); this was due to the different experimental conditions in both systems, mainly to the greater amount of diet supplied to *CF*. Solids retention time was also greater (P<0.05) in *Rusitec* than in *CF* (48 vs 30 h), and this could have contributed to the greater (P<0.05) efficiency of microbial growth found in *CF,* as longer solids retention time tends to decrease cell growth rate (Veldkamp, 1976). Values of EMG were in the range of those previously reported for similar diets in sheep (Archimède *et al.*, 1997), *CF* (Meng *et al.*, 1999) and *Rusitec* (Carro and Miller, 1999). Bacterial

cell yields and efficiency of growth depend on cell growth rate and maintenance requirements (Hespell and Bryant, 1979), and maintenance requirements vary with bacterial species and environmental conditions, such as pH, dilution rate, solids retention time, etc (Hoover *et al.*, 1984; Meng *et al.*, 1999).

Conclusions

For most parameters measured, there were differences between the fermentation in sheep rumen and *in vitro* systems. Some of these discrepancies can be attributed mainly to observed differences in pH values, solids retention time and fractional outflow rate of liquid phase, as well as to lack of absorption from the fermenters. A more precise control of pH and liquid dilution rate in both types of fermenters could probably improve the simulation of the *in vivo* fermentation.

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