Original article

Quantitative effects of alfalfa extract supply on rice straw degradation, fermentation and biomass synthesis by rumen microorganisms in vitro

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Abstract — In Asia and Africa, rice straw enters into livestock feeds as the main constituent. It is sometimes associated with fresh legume material, from local garden farming or from legume-cereal rotations, but the possible benefits of this practice need to be estimated more precisely, with special attention to the influence of cytoplasmic contents other than nitrogen or macrominerals on rumen microbial metabolism. In the present study, fresh *Medicago sativa* extract (AE) was chosen as a model of cytoplasmic contents from tops of tropical legumes such as *Vigna unguiculata*. It was obtained from whole plants harvested at the beginning of flowering (primary growth). The effects of AE supplementation on a rumen microbial community maintained on a diet made of rice straw and inorganic nitrogen (CP: 73.3 g·kg^{-1} DM) were assessed in dual outflow continuous culture. Three input levels were applied in order to detect curvilinear effects: 0, 0.227 and 0.455 ml·g^{-1} straw DM. The pH and the redox potential varied from 6.72 to 6.84, and from –335 to –370 mV respectively. The addition of AE had minor effects on fermentation variates. The true degradability of dietary OM and the degradabilities of ash-free NDF and ADF were not modified by AE. Alfalfa extract lowered the outflow of fermented OM (FOM) by 14% while it enhanced the microbial OM outflow (+33%) and the efficiency of microbial protein synthesis (15 to 26 g N·kg^{-1} FOM). This effect was curvilinear, and appeared negligible above 45–50% of the maximal extract input rate. This legitimised the addition of fresh legume materials to the diet even in small amounts.

rumen / rice straw / digestion / protein synthesis / in vitro

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Résumé — **Les effets quantitatifs de l'apport d'extrait de luzerne sur la dégradation de la paille de riz, la fermentation et la synthèse de biomasse par les micro-organismes du rumen in vitro.** En Asie et en Afrique, l'affouragement des ruminants en paille de riz est parfois associé à des déchets de maraîchage ou à des légumineuses (Niébé). Les bénéfices éventuels de cette pratique doivent être vérifiés, en portant une attention particulière à l'action des composés cytoplasmiques autres que les protéines et les minéraux majeurs sur le métabolisme des micro-organismes du rumen. Le contenu cytoplasmique du modèle choisi est un extrait par pressage d'une luzerne (*Medicago sativa*) récoltée en début de floraison (EL). Nous avons étudié l'effet de l'EL sur une communauté de micro-organismes du rumen maintenue en fermenteurs à double effluent sur une ration paille de riz + azote inorganique (MAT : 73.3 g·kg^{-1} MS). Trois niveaux d'apport ont été appliqués pour détecter d'éventuels effets curvilinéaires : 0 ; 0,227 ; 0,455 ml·g^{-1} MS paille. Le pH et le potentiel redox ont varié de 6,72 à 6,84, et de –335 à –370 mV respectivement. L'EL n'a pas significativement modifié les variables fermentaires, ni la dégradabilité réelle de la MO, ou les dégradabilités du NDF et ADF déminéralisés. L'extrait de luzerne a diminué le flux de MO fermentée (MOF) de 14 % tandis qu'il a accru le flux de MO microbienne (+ 33 %) et l'efficacité de la synthèse de protéines microbiennes (de 15 à 26 g N·kg^{-1} MOF). Cet effet a été curvilinéaire et paraît négligeable au delà de 45–50 % du niveau d'apport maximal. Dans notre essai, l'EL n'a pas significativement modifié l'apport d'énergie et d'azote aux micro-organismes. Son action catalytique sur le métabolisme microbien justifie l'apport de matière végétale fraîche dans la ration même en faible quantité.

rumen / paille de riz / digestion / synthèse protéique / in vitro

1. INTRODUCTION

Assessing nutritional strategies for improving rice straw utilisation by ruminants remains a challenging goal in developing countries. The approximate annual world production of rice straw can be estimated from the rice production figures (408 Mt for 1999/2000 according to the USDA-Foreign Agricultural Service) at 400–800 Mt per year, as the ratio of grain to grain plus straw equals 0.50 with modern varieties and 0.3 with traditional ones [19]. This represents an amount of biomass equivalent to wheat straw. In western countries, where less than 3% of the rice is produced, rice straw is merely used to provide the dietary fibre fraction necessary for proper functioning of the rumen, most of the digestible energy being brought into the diet by cereals and other concentrates [19]. In Asia and Africa, this roughage enters into livestock feeds as the main constituent. However, when fed alone on a long-term basis, this low-quality roughage is insufficient to meet the animal's maintenance needs. It may then be mixed with fresh plant material, legumes or wastes from vegetables issued from local garden farming. In Sudan, cowpea tops (*Vigna unguiculata)* have been commercialised as feed supplements for cattle [33]. This legume has also been successfully grown in rotation with Pearl millet in India [15] and a number of cereals in Nigeria [14, 29]. Similarly, *Sesbania rostrata* and *Dolichos lablab* have been associated with rice, sorghum and millet in Africa [1]. However, the possible benefits of this practice need to be estimated precisely, with careful attention to the specific influence of plant cytoplasmic compounds other than nitrogen and macrominerals on rumen microbial metabolism.

The aim of the present study was to characterise and quantitate, in vitro, the effects of fresh legume extract supply on the metabolism of a rumen microbial community maintained on a diet made of rice straw and inorganic nitrogen. The use of dual outflow fermenters allowed the simultaneous observation of the degradation of major feed constituents, individual gas and net volatile fatty acid productions, and biomass synthesis by micro-organisms.

2. MATERIALS AND METHODS

2.1. Experimental scheme

Alfalfa extract was chosen as a model of cytoplasmic contents because its biochemical composition was similar to *Vigna unguiculata* from which fresh material was not available in France. Both species exhibit similar ranges of variation for niacin, riboflavin, thiamin, β-carotene and mineral concentrations in aerial parts [11]. The main difference is the concentration of ascorbic acid which is a factor of three smaller in *Vigna unguiculata* than in alfalfa. Alfalfa extract did not interfere with the measurement of straw cell-wall fraction degradabilities. Its contribution to the inflow of cell-wall constituents was negligible, below the quantification level permitted by the gravimetric measurements in the NDF-ADF fractionation method.

As the possible effects of legume supplementation were not characterised in the literature, three extract input levels were applied in order to detect curvilinear effects: 0, 0.227 and 0.455 ml·g^{-1} straw dry matter (DM). The supplementation of rice straw with alfalfa extract corresponded to an incorporation level of whole alfalfa in the diet up to 10% on a DM basis. The study totalled 12 runs, in 3 seven-day consecutive experimental periods. For each period, the extract inputs were randomly assigned to 3 dual outflow fermenters (working volume of 1.1 litre). On period 3, three test-runs with intermediate alfalfa amounts (one at 0.114 ml·g^{-1} straw DM and two at 0.341 ml·g^{-1} straw DM) were performed in parallel with the main trial on additional fermenters, to assess the choice of second order polynomials as models of the effects of alfalfa on rumen microorganisms metabolism. The data collected at these test-runs did not participate in the estimation of regression weights.

2.2. Experimental feeds

The rice straw (*Oryza sativa*, variety Thaibonnet) was harvested in the Camargue (Rhône delta) region of France. It was coarsely ground (screen aperture of 8 mm) and pelleted (Unité de Préparation des Aliments Expérimentaux, INRA Jouy-en-Josas, France). The DM content was 858 g·kg^{-1} FM, and DM composition was (g·kg^{-1} DM): organic matter (OM) 888, neutral detergent fiber (NDF) 733, acid detergent fiber (ADF) 428, acid detergent lignin (ADL) 65, crude protein 36.9.

The alfalfa (*Medicago sativa*) extract was obtained from whole plants harvested at the beginning of flowering (first growth), coarsely chopped and divided into portions of 200 g. These long chop batches were immediately stored in air-tight plastic bags, frozen and kept at –20 °C until used. The soluble sugars and crude protein (determined by the Dumas technique) contents of the extract were 12.0 g·l^{-1} and 15.9 g·l^{-1} respectively. Fresh extract was prepared every incubation day. At 9.00 h, a portion of alfalfa was defrosted and finely chopped in a blender. The extract was squeezed from chopped alfalfa in a stainless steal mechanical press at a rate of 61% of initial water content. It was kept at +4 °C until it was introduced into the fermenters.

2.3. Incubation procedure

The three donor animals were ruminally-cannulated wethers fed 1 200 g·d^{-1} rice straw and 100 g·d^{-1} soybean meal in two meals with free access to water. The rumen contents used as inoculum were taken after a 24 h fasting period and processed as in [6]. Each fermenter was maintained at 39 °C and was continuously infused with artificial saliva whose composition is detailed in [5]. This buffer contained 0.4 g·l^{-1} HCl-Cysteine as a reducing agent. The fermentation broths were separately supplemented with 88 mg·d^{-1} CaCl$_2$ 2H$_2$O, 42 mg·d^{-1} MgCl$_2$

4.5 H_2O. Eleven grams of pelleted straw (on a DM basis) and 5 ml of a 30.57 g·l^{-1} NH$_4$Cl solution was supplied to the fermenters at 11.00 h and 23.00 h. At the same time, the alfalfa extract prepared at 9.00 h was pipetted into the fermenters (1.25, 2.5, 3.75 or 5 ml per meal). The in vitro dilution rates of particle and liquid phases were set at 0.03·h^{-1} and 0.06·h^{-1}, respectively. The procedures followed to control the two turnover rates, and for the daily collection of displaced and filtered effluents, were identical to [6].

2.4. Analytical methods

After a five-day adaptation term, 10 ml of filtered (pore size of the filters = 200 μm) fermentation broth were taken at 10.00 h for redox potential and pH measurement, and subsampled for volatile fatty acids (VFA), ammonia nitrogen (NH$_3$-N) and protozoa population density determination. Protozoa were counted according to [6]. The fermentation gas volume and composition were determined on days 6 and 7 by gas chromatography as described in [4]. The displaced and filtered effluents collected on days 6 and 7 were pooled and subsampled for DM, VFA and NH$_3$-N analysis. The samples for VFA and NH$_3$-N determination were mixed with 0.1 volume of H$_3$PO$_4$ 8.2% (w/w) and stored at –20 °C until processing. NH$_3$-N was determined as described by [9]. VFA were determined as described in [3].

On day 7, the bacterial reference pool for proteosynthesis determination was isolated from the pooled effluents by the following procedure. One litre of fresh total effluent per vessel was adjusted to pH 8.0 with 1N NaOH, and homogenised for 3 × 1 min in a Waring blender, according to [35]. The feed residue was then separated by centrifugation at 1 500 g for 10 min. The supernatant was centrifuged at 20 000 g for 30 min to isolate the bacterial fraction. The bacterial pellet was resuspended in NaCl

(9 g·l^{-1}), centrifuged at 20 000 g for 30 min and freeze-dried. The remaining effluents were also freeze-dried. Feeds and freeze-dried effluents were ground prior to analysis using a Culatti grinder with a screen of 0.8 mm aperture. Feed, effluent and bacterial samples were analysed for DM, OM [7], NDF and ADF [36]. Total nitrogen was measured by the Dumas technique. Effluent and bacterial samples were analysed for nucleobases, used as microbial markers, according to [21] using a diode-array detector (Beckman Instruments, Fullerton).

2.5. Calculations and statistical analyses

The daily amount of fermented OM (FOM) was estimated from the relation given by Demeyer and Van Nevel [10] to calculate the amounts of hexoses theoretically fermented (HF).

$$HF \ (mol·d^{-1}) = (C2 + C3)/2 + C4 + C5 \ (1)$$
$$FOM \ (g·d^{-1}) \ = 162 \times HF \quad\quad (2)$$

where C2, C3, C4, and C5 were the daily outflows (mol·d^{-1}) of acetate, propionate, butyrate and valerate, respectively. The true OM degradability (td$_{OM}$) was determined from dietary OM inflow and fermented and microbial OM outflows as detailed in [4]. The degradabilities of ash-free NDF and ADF were calculated from the determination of their ash content in quadruplet. The efficiency of microbial protein synthesis (EMPS) was determined by equation (3).

$$EMPS = \text{g microbial N daily outflow /}$$
$$\text{kg FOM daily outflow.} \quad (3)$$

The results were submitted to a mixed-model analysis of covariance (ANCOVA) using the MINITAB GLM procedure [22, 34]. Terms in the model were alfalfa supplementation (Su) and period (Pe). The period factor was random and the alfalfa factor was the covariate. Alfalfa sums of squares were partitioned into linear and quadratic effects. The variable Su was

coded: it varied from –1 to +1 and was related to the actual amount of alfalfa (A) supplied (in ml per g straw DM), by the equation:

$$Su = A / 0.227 - 1. \qquad (4)$$

For any response, the predicted value Y for a given level of alfalfa supplementation can be calculated using the corresponding regression weights estimates and the coded variable Su:

$$Y = b_0 + b_1 \times Su + b_2 \times Su^2. \qquad (5)$$

As an illustration, when alfalfa extract was supplemented at maximal rate, the variable Su was equal to +1 and, according to the coefficients given in Table I the predicted pH equalled:

$$pH = 6.76 - 0.03 \times Su + 0.01 \times Su^2$$
$$= 6.76 - 0.03 + 0.01 = 6.74. \qquad (6)$$

The adequacy of second order polynomials to model the action of alfalfa supplementation was preliminarily tested by comparing predicted response values with measured values at three test-runs in period 3, and required to estimate the effect of this specific period on responses. Thus, the experimental results were submitted to multiple linear regression by a MINITAB procedure [22]. The prediction interval for an individual observation was estimated with a P-value of 95%. The tables only summarise the results of mixed model ANCOVA.

3. RESULTS

Although three test runs constituted a limited validation, their data did not belie the use of second-order polynomials to model the influence of alfalfa extract on the metabolic activity of rumen micro-organisms maintained on a rice straw diet as, for most variates, all three values measured in

Table I. ANCOVA results for fermentation pattern.

Response		pH	Eh	Protozoa (μl^{-1})		VFA	Molar proportion (%VFA)			NH_3-N
			(mV)	>70 μm	Total	(mM)	C2	C3	C4	mg·l^{-1}
Source	d.f.	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$
Su	1	0.045	0.34	0.13	0.59	0.93	0.38	0.13	0.21	0.12
Su^2	1	0.65	0.96	0.51	0.075	0.83	0.90	0.51	0.20	0.51
Pe	2	0.098	0.10	0.087	0.093	0.23	0.19	0.56	0.09	0.002
RSD	4	0.024	7.88	0.521	5.65	5.59	1.02	0.910	0.436	2.73
Regression weights										
b$_0$		6.76	–346.2	2.33	16.5	56.3	70.8	20.8	7.17	14.0
		(± 0.014)	(± 4.5)	(± 0.30)	(± 3.3)	(± 3.2)	(± 0.59)	(± 0.53)	(± 0.25)	(± 1.6)
b$_1$		–0.03	3.4	0.40	–1.3	0.20	0.41	–0.70	0.26	2.2
		(± 0.010)	(± 3.2)	(± 0.21)	(± 2.3)	(± 2.3)	(± 0.42)	(± 0.37)	(± 0.18)	(± 1.1)
b$_2$		0.01	–0.3	–0.27	9.5	–0.93	–0.10	0.47	–0.47	–1.4
		(± 0.017)	(± 5.6)	(± 0.37)	(± 4.0)	(± 4.0)	(± 0.72)	(± 0.64)	(± 0.31)	(± 1.9)
R^2		0.81	0.71	0.77	0.79	0.53	0.61	0.58	0.78	0.96

Eh: redox potential, VFA: volatile fatty acids, NH_3-N: ammonia-nitrogen, C2: acetate, C3: propionate, C4: butyrate, $P > F$: probability of variance ratio exceeding the tabulated F-value, RSD: residual standard deviation. Regression model: Response = $b_0 + b_1 Su + b_2 Su^2$.

these test-runs were in accordance with predictions. Only 4 values were out of their prediction interval from a total of 99, which is statistically expected. They concerned rather minor variates: two associated with the population density of protozoa and two with the productions of isobutyrate and valerate.

3.1. Characteristics of fermentation broths

The variates related to fermentation broths at 10.00 h are given in Table I. The addition of alfalfa extract had minor effects. The pH and the redox potential varied from 6.72 to 6.84, and –334.9 to –369.2 mV, respectively, and both ranges were optimal with regard to rumen microbial metabolism. The VFA concentration in the fermentation broths ranged between 45.9 and 63.4 mM. The fermentation pattern was characterised

by low levels of butyrate. Protozoa numbers varied from 8.4 to 37.2 μl^{-1} (and from 1.0 to 3.2 μl^{-1} for large species). Alfalfa supplementation linearly influenced a number of variates: it tended to acidify the fermentation broth, to favour large protozoan species (+48% from 0 to 10 ml·d^{-1} extract), to decrease propionate molar proportion (–6%) and to increase NH_3-N concentration (+42%).

3.2. Energy metabolism

The true degradability of dietary OM and the degradabilities of ash-free NDF and ADF ranged from 42.9 to 51.6%, 34.4 to 53.5% and 32.5 to 54.0%, respectively. They were not modified by alfalfa supplementation. On the contrary, the addition of alfalfa extract altered the extent of fermentation (Tab. II). The input of 10 ml·d^{-1} extract

Table II. ANCOVA results for volatile fatty acid production, daily amounts of hexoses theoretically fermented (HF) (mmol·d^{-1}), and relative productions of acetate (C2), propionate (C3) and butyrate (C4) (mol·100 mol^{-1} HF).

Response	C2	C3	IC4	C4	IC5	C5	HF	C2	C3	C4
				(mmol·d^{-1})					(mol·100 mol^{-1} HF)	
Source	d.f.	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$
Su	1	0.095	0.16	0.12	0.92	0.55	0.024	0.11	0.38	0.62	0.51
Su^2	1	0.26	0.80	0.31	0.26	0.34	0.22	0.61	0.16	0.15	0.17
Pe	2	0.78	0.77	0.30	0.64	0.11	0.001	0.88	0.57	0.38	0.59
RSD	4	6.23	1.53	0.067	1.30	0.024	0.014	3.92	6.66	1.56	2.66
Regression weights											
b$_0$		53.9	17.4	0.164	7.0	0.170	0.490	43.7	123.2	39.8	16.0
		(± 3.6)	(± 0.88)	(± 0.039)	(± 0.75)	(± 0.014)	(± 0.008)	(± 2.3)	(± 3.8)	(± 0.90)	(± 0.1.5)
b$_1$		–5.5	–1.1	0.054	–0.1	0.007	0.020	–3.3	–2.7	0.3	0.8
		(± 2.5)	(± 0.62)	(± 0.027)	(± 0.53)	(± 0.010)	(± 0.006)	(± 1.6)	(± 2.7)	(± 0.64)	(± 1.1)
b$_2$		5.7	–0.3	0.055	–1.2	–0.018	–0.014	1.5	8.3	–2.0	–3.2
		(± 4.4)	(± 1.1)	(± 0.047)	(± 0.92)	(± 0.017)	(± 0.010)	(± 2.8)	(± 4.7)	(± 1.1)	(± 1.9)
R^2		0.64	0.48	0.68	0.41	0.71	0.97	0.55	0.57	0.60	0.53

$P > F$: probability of variance ratio exceeding the tabulated F-value, RD: residual standard deviation. Regression model: Response = b$_0$ + b$_1$ Su + b$_2$ Su^2.

lowered daily amounts of hexoses fermented by 14%, mostly through a negative linear influence on the production rate of C2 (–17%). Similarly, this input increased the outflow of minor VFA, isobutyrate (IC4, +65%) and C5 (+9%). When expressed in terms of moles per 100 moles of hexoses fermented, the production of the three major VFA was slightly affected by alfalfa in a quadratic manner, the relative output of C2 being minimal and those of C3 and C4 being maximal for intermediate supplies of extract. The output of fermentation gases was not significantly modified by the addition of alfalfa extract, as shown in Table III. Carbon dioxide relative production tended to increase, possibly because of the slight acidification of fermentation broths.

3.3. Biomass synthesis

The variates linked to microbial biomass synthesis are presented in Table IV. The

Table III. ANCOVA results for gas production.

Response		Volume	CO_2	CH_4
		$(l \cdot d^{-1})$	$(mol \cdot 100 \; mol^{-1} \; HF)$	
Source	d.f.	$P > F$	$P > F$	$P > F$
Su	1	0.20	0.086	0.11
Su^2	1	0.17	0.41	0.48
Pe	2	0.047	0.24	0.20
RSD	4	0.328	34.2	9.93
Regression weights				
b_0		3.30	241	64.4
		(± 0.19)	(± 19)	(± 5.7)
b_1		0.21	32	8.4
		(± 0.13)	(± 14)	(± 4.1)
b_2		0.39	22	5.5
		(± 0.23)	(± 24)	(± 7.0)
R^2		0.83	0.72	0.71

$P > F$: probability of variance ratio exceeding the tabulated F-value, RSD: residual standard deviation. Regression model: Response = $b_0 + b_1 \, Su + b_2 \, Su^2$.

Table IV. ANCOVA results for fermented organic matter (FOM) and microbial organic matter outflows (MOM) (g·d^{-1}), true degradability of OM (tdOM), OM partitioning into fermented (Omf) and microbial (OMm) fractions, ash-free NDF and ADF degradabilities, microbial nitrogen daily outflow (MN), and efficiency of microbial protein synthesis (EMPS) (g N·kg^{-1} FOM).

Response		FOM	MOM	tdOM	OMf	OMm	dNDF	dADF	MN	EMPS
		g·d^{-1}				%			mg·d^{-1}	
Source	d.f.	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$	$P > F$
Su	1	0.11	0.008	0.43	0.09	0.009	0.71	0.79	0.038	0.008
Su^2	1	0.61	0.20	0.89	0.60	0.20	0.74	0.70	0.083	0.057
Pe	2	0.87	0.20	0.86	0.88	0.19	0.58	0.74	0.18	0.16
RSD	4	0.636	0.164	3.67	3.16	0.811	7.13	7.91	10.2	1.58
Regression weights										
b_0		7.08	2.46	47.4	35.2	12.20	46.2	46.4	162.7	23.1
		(± 0.37)	(± 0.095)	(± 2.1)	(± 1.8)	(± 0.47)	(± 4.1)	(± 4.6)	(± 5.9)	(± 0.91)
b_1		–0.53	0.33	–1.3	–2.9	1.57	–1.2	–0.9	12.7	3.2
		(± 0.26)	(± 0.067)	(± 1.5)	(± 1.3)	(± 0.33)	(± 2.9)	(± 3.2)	(± 4.2)	(± 0.65)
b_2		0.25	–0.18	0.4	1.3	–0.89	–1.8	–2.3	–16.6	–3.0
		(± 0.45)	(± 0.12)	(± 2.6)	(± 2.2)	(± 0.57)	(± 5.0)	(± 5.6)	(± 7.2)	(± 1.1)
R^2		0.55	0.89	0.21	0.58	0.88	0.28	0.18	0.83	0.90

$P > F$: probability of variance ratio exceeding the tabulated F-value, RSD: residual standard deviation. Regression model: Response = $b_0 + b_1 \, Su + b_2 \, Su^2$.

supplementation of fermentation broth with alfalfa extract lowered the outflow of fermented OM (–14%) and enhanced the outflow of microbial OM (+33%), as shown in Figure 1. This effect was curvilinear, with a decreasing marginal efficiency of alfalfa extract. According to the model, the first millilitre of extract increased the microbial OM output by 0.13 g·d^{-1} while the last millilitre increased output only by 0.01 g·d^{-1}. The addition of alfalfa also shifted the partition of dietary OM in favour of microbial OM (OMm), to the detriment of fermented OM (Omf), with no significant variation of undegraded dietary OM fraction.

The outflow of microbial N and the EMPS varied from 124 to 174 mg·d^{-1} and from 15.2 to 26.4 g N·kg^{-1} FOM, respectively. Both variates were increased by alfalfa supplementation in a curvilinear way. According to the model, the EMPS was equal to 17 g N·kg^{-1} FOM in the absence of alfalfa. It reached a maximal value of 24 g N·kg^{-1} FOM when the extract input rate equalled 7.6 ml·d^{-1}, the first 2.5 ml·d^{-1} accounting for 55% in the increase of EPMS. As shown by Figure 2, this can be interpreted in biological terms as a plateau reached at approximately 50% of the maximal extract input rate.

4. DISCUSSION

The introduction of alfalfa extract into the continuous culture systems induced unequivocal changes in a number of microbial functions, which highlighted the mode of action of cytoplasmic contents on the metabolism of rumen microbes maintained on rice straw.

The OM and fibre degradabilities measured with the basal diet belonged to the upper part of the physiological range of variation reported in the literature. For example, NDF and ADF digestibilities of 56.5% and 51.8%, respectively, were recorded in Ongole × Holstein dairy cows fed with rice straw ad libitum and 1 kg·d^{-1} concentrate [27], while NDF and ADF digestibilities of 73% were observed in Zebu cows receiving a 80% rice straw: 20% cotton seed meal diet at maintenance level [16]. However, our results were consistent with previous
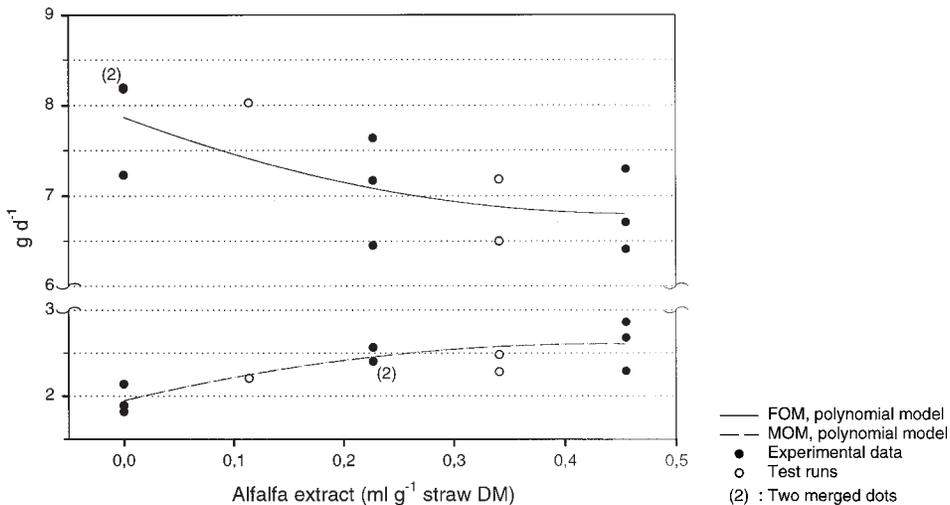
Figure 1. Effect of alfalfa extract on the daily outflows of fermented OM (FOM) and microbial OM (MOM).
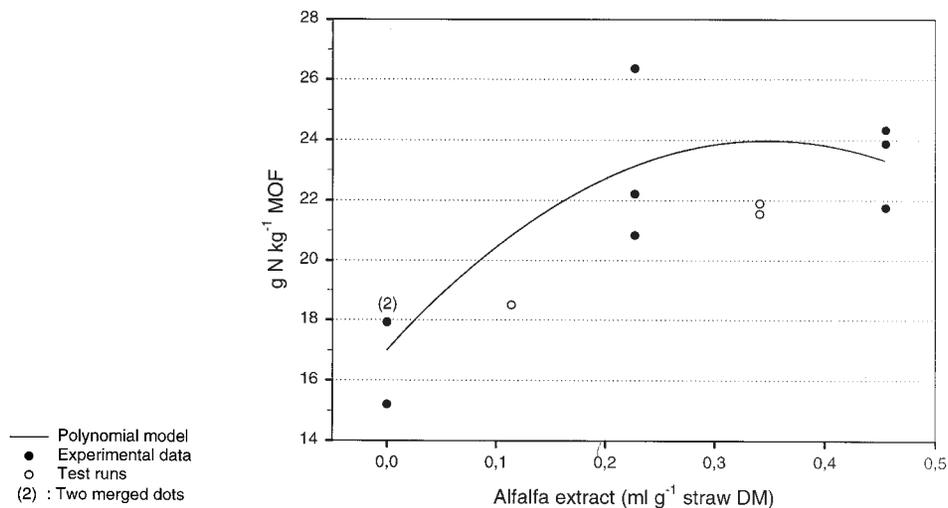
Figure 2. Effect of alfalfa extract on the efficiency of microbial protein synthesis.

reports of in vitro experiments. Using a chemostat culture set at a dilution rate of $0.055 \ h^{-1}$ and fed with a diet made of untreated rice straw: concentrate 75:25, Karunanandaa and Varga measured NDF and ADF degradabilities of 48.0% and 49.4% respectively [20]. The straw used in the present work had a composition close to the INRA standard values (in $g \cdot kg^{-1}$ DM, OM 850, CP 37, cellulose 367) [2], and within the range of variation reported in the literature [19]. One can only notice the rather low ash content (11.2% DM), as this constituent varies from 10.4 to 21.8% DM, with an average value of 16.2% DM [19]. The high degradability of our straw can be related to two physical characteristics. First, visual inspection of the bales showed that part of the leaves had been lost, probably during raking and baling. The resulting enrichment in culm fraction was consistent with the low mineral content observed as, for rice straw, ash and silica contents in leaves can reach values twice as large as in culms [17, 30, 32]. The reduced leafiness was susceptible to improve the degradability of cell-wall constituents because high levels of silica were associated with lower

fibre digestibility [18, 19]. Moreover, the preliminary grinding and pelleting of straw, which is a necessary process in experiments involving dual outflow fermenters, increased the number of attachment sites for fibre-degrading bacteria and was likely to favour bacterial colonisation of straw as this process was to take place on substrate surface, unlike fungal attack which would preferentially progress within the inner layers of roughage biomass [24].

Supplementing rice straw with alfalfa extract did not change the extent of degradation of OM or fibre. One must stress that the additional amounts of non structural sugars supplied by alfalfa extract can be considered as negligible. It was estimated from published data on alfalfa intracellular carbohydrates [8] that at its maximal rate, alfalfa supplementation supplied the fermentation broth with only 0.4 $g \cdot d^{-1}$ starch, 0.1 $g \cdot d^{-1}$ sucrose and 0.06 $g \cdot d^{-1}$ glucose. Besides, straw cell-wall degradation may have already been maximised by the above-mentioned physical characteristics. Alfalfa supplementation may also have enhanced initial fibre hydrolysis rates without

significantly modifying the overall extent of fibre degradation. As a matter of fact, a lack of improvement of in sacco rumen degradability of rice straw DM in response to supplementation of buffaloes diet with legume straws or protein meals for incubations times exceeding 24 h has been repeatedly reported [28].

Ammonia concentration in fermenters was quite low, but was consistent with in vivo data [23]. The highest level of alfalfa extract supplementation increased NH_3-N concentration from 10.4 to 14.8 mg·l^{-1}. The latter value was close to the optimal threshold of 16 mg·l^{-1} reported to maximise barley straw digestion [25], but we did not observe any significant change in straw cell wall degradation in the present study. Thus, ammonia concentration did not appear to be a limiting factor for microbial metabolism, even for fibrolytic activity, which is known to be sensitive to the supply of ammonia [12]. More generally, the nitrogen content of our experimental diets varied within a narrow range, from 7.33 to 7.94% CP. Most of this dietary N (5.89 to 6.52% CP) was readily available for microbes. This clearly shows that alfalfa extracts did not dramatically change nitrogen supply to microorganisms.

Adding alfalfa extract strongly lowered the net outflows of fermented organic matter and resulted in higher microbial biomass yields. We have shown that alfalfa did not significantly modify N and energy supply to the microbial population in fermenters. Besides, the amounts of phosphorus and sulphur provided by artificial saliva and cysteine were sufficient to meet the microbial metabolic requirements [13]. As the release of hexoses through structural carbohydrate degradation remained unchanged, alfalfa probably restrained the extent of microbial biomass recycling in the fermenters by directly supplying molecules of high metabolic value, such as limiting amino acids, growth factors or coenzymes. When the fermenters were fed on the basal diet,

these growth factors had to be synthesised within the microbial community and required the autolysis of a portion of the rumen microbial population to support the completion of the growth cycle of the remaining living microorganisms. Published data on comparable trials are scarce. In an assay implementing the chemostat technique (which means equal solid and liquid retention times), the variates linked to fermentation and biomass synthesis rates from a rice straw: concentrate 75:25 diet (11.2% CP) clearly had lower values than in the present work. In particular, the EMPS equalled 16.2 g N·kg^{-1} FOM. Identifying the possible causes of these differences is uneasy. Both assays implemented similar microbial markers, nucleobases in our case and purines in [20]. In our study, the use of nucleobases was justified by the low input of nucleic acids of straw origin and the great susceptibility of alfalfa extract nucleic acids to enzymatic lysis, as suggested by the way MOM and EMPS reacted to alfalfa supplementation. Moreover, taking into account the protozoa bio-volumes, chemical composition and outflow rate reviewed by Williams and Coleman [37], one can estimate from protozoa counts that these microorganisms accounted for less than 2% of MOM. Thus, the possible discrepancies between the protozoal biomass and microbial reference pools in terms of OM and nucleobase concentrations cannot invalidate our conclusions. A higher EMPS in [20] would also have been expected owing to the shorter retention time for solids (18 h compared to 33 h in our case), in accordance with chemostat theory, and also to the higher NH_3-N concentration, along with the dogma of a minimal value of 50–80 mg NH_3-N·l^{-1} for optimal proteosynthesis [31]. The diet in [20] was more elaborate than our basal treatment as it contained soy bean meal (14% DM), a trace mineral premix and a mixture of vitamins A, D and E. Besides the possible consequences of the short straw fibre retention time on the microbial specific composition, these discrepancies may

partly stress the nutritional importance of additional growth factors present in green plant material.

The most interesting outcome of the present assay is the decreasing marginal efficiency of alfalfa extract on biomass synthesis. In the same way, Prakash et al. investigated the catalytic action of 100 g·d^{-1} protein meals on the digestion of rice straw-poultry droppings-rice bran diets by buffaloes [26]. They attributed the higher N retention and nutrient utilisation with protein meal supplemented diets to a better conversion of rumen ammonia into microbial protein. Our observations confirmed this hypothesis. In our case, this type of relationship between plant extract supplementation and microbial activity legitimised the addition of fresh materials to the diet even in small amounts.

ACKNOWLEDGMENTS

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