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In situ study of the relevance of bacterial adherence to feed particles for the contamination and accuracy of rumen degradability estimates for feeds of vegetable origin

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An *in situ* study was conducted on four rumen-cannulated wethers to determine (using ^{15}N infusion techniques) the microbial contamination (mg bacterial DM or crude protein (CP)/100 mg DM or CP) and the associated error on the effective degradability of fourteen feeds: barley and maize grains, soyabean and sunflower meals, full-fat soyabean, maize gluten feed, soyabean hulls, brewers dried grains, sugarbeet pulp, wheat bran, lucerne and vetch-oat hays, and barley and lentil straws. The DM or CP contamination in residues (M) fitted to single exponential or sigmoid curves. A general model ($M = m\ (1 - e^{-ft})^j$) was proposed to match this fit. Asymptotic values (m) varied from 2·84 % to 13·3 % and from 2·85 % to 80·9 % for DM and CP, respectively. Uncorrected results underestimated the effective degradability of both DM ($P < 0·05$) and CP ($P < 0·01$). For CP, this underestimation varied from 0·59 % to 13·1 %, with a higher but unascertainable error for barley straw. Excluding maize grain, the microbial contamination of both DM and CP, and the associated underestimation of the effective degradability of CP, were positively related to the cellulose content of the feed. The error in the effective degradability of CP was also negatively related to the CP content and its apparent effective degradability (R^2 0·867). This equation allows easier and more accurate estimates of effective degradability, needed to improve protein-rationing systems.

Rumen: Microbial contamination: Effective degradability: ^{15}N/N: Sheep

The current systems of protein rationing for ruminants require an accurate knowledge of the quantity of the microbial and feed proteins that reach the post-ruminal tract. The magnitude of both protein fractions is influenced by the rumen degradation of feeds included in the ration. *In situ* methods are the most common way to obtain estimates of effective degradability (ED). However, adherent micro-organisms that colonise and degrade feed particles simultaneously contaminate them. The resultant underestimate of the ED of crude protein (CP) or amino acids in vegetable feeds may be important as a consequence of the high N content in micro-organisms, although feed characteristics determining microbial adherence and the development and persistence of the micro-organisms may also influence this underestimate.

In spite of these errors, uncorrected ED values are commonly employed in current systems, because the estimation of microbial contamination is a laborious, complex and expensive procedure. Although microbial attachment has been the subject of many types of research, quantitative and systematic *in situ* studies across a wide range of feeds are scarce (Michalet-Doreau & Ould Bah, 1989), and in most cases these have focused only on the effects of N contamination on ED estimates. Studies on microbial colonisation may, however, also contribute to a better knowledge of factors affecting feed degradation (McAllister *et al.* 1994) and microbial protein supply associated with feed particles, which represents an important contribution to the nutrition of the ruminant (Merry & McAllan, 1983, Rodríguez *et al.* 2003). The objectives of the present work were to determine the microbial contamination of feeds of vegetable origin in the rumen and the associated underestimation of ED, as well as to develop systems to predict these errors.

Materials and methods

Animals and feeding

Four wethers (average body weight 62·2 kg) equipped with detachable rumen cannulae (inner diameter 80 mm) were fed a diet composed of chopped vetch-oat hay and concentrate (2:1, w/w on a DM basis). The concentrate contained (g/kg): maize grain (CG) 607; dehydrated beet pulp 300; soyabean meal 45; fish meal 20; bentonite 15; minerals and vitamins 13. The diet was offered as six meals/d, at intervals of 4 h, at a rate of intake of 40 g DM/kg $W^{0·75}$, equivalent to 1·1 and 1·6 times the maintenance requirements for energy and N, respectively. The chemical composition of hay and concentrate was described in a previous study (Rodríguez *et al.* 2000).

Abbreviations: ADF, acid detergent fibre; CG, corn grain; CGF, corn gluten feed; CP, crude protein; ED, effective degradability; NDF, neutral detergent fibre; SAB, solid adherent bacteria; SBH, soyabean hulls; SBM, soyabean meal; SBP, sugarbeet pulp.

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Experimental procedures

After an adaptation period to the diet of 14 d, fourteen different feeds were incubated in nylon bags in the rumen (barley grain, CG, sunflower and soyabean (SBM) meals, full-fat soyabean, maize gluten feed (CGF), soyabean hulls (SBH), brewers dried grains, sugarbeet pulp (SBP), wheat bran, lucerne hay, vetch-oat hay, and barley and lentil straws. Starting 3 d before the *in situ* study and through all the experiment, a ^{15}N-enriched $(NH_4)_2SO_4$ solution was infused at a rate of 40 mg ^{15}N/d to label the rumen bacteria (for more details of infusion, see Rodriguez *et al.* 2003). Feeds were characterised (Table 1) by their chemical composition and cell wall constituents as neutral and acid detergent fibre (NDF and ADF, respectively), acid detergent lignin and the proportions of insoluble N in NDF and ADF solutions. The isotopic ratio ^{15}N:N (^{15}N/(^{14}N + ^{15}N)) was determined and compared in the whole feed and in its insoluble fraction (0 h residues; see later).

Feed samples of approximately 3 g (air dry basis) were incubated in the rumen for periods of 2, 4, 8, 16, 24 and 48 h plus an additional time of 72 h in forages and fibre byproducts. Bags measuring 11 cm × 7 cm (inner dimensions) were made by heat-sealing (Preci-Pack P30N; Dover Pack S.A., Barcelona, Spain) from nylon cloth with a pore size of 46 μm (reference 120T; Tissages Tissues Techniques, Villeneuve la Garenne, France). Two series of incubations were conducted for each animal on different days for each feed. At each series of incubation, all bags of the different incubation times (one bag per time) of the feed tested were placed simultaneously in the rumen just before the sheep were offered their first meal of the morning. After collecting from the rumen, bags were washed with tap water and stored at −20°C. When thawed, the bags were washed three times for 5 min in a turbine washing machine. This same washing procedure was applied to three bags of each feed to obtain the 0 h value.

Bags were then dried for 48 h at 80°C in an oven and feed residues analysed for DM, N and ^{15}N:N. The proportion of microbial DM or N in the incubated residues was determined from the ^{15}N enrichment of these residues and of the rumen solid adherent bacteria (SAB), which were isolated from each animal at the end of the experiment. Values for ^{15}N abundance and N content of the SAB samples were previously described (Rodriguez *et al.* 2000). The microbial N content of the bag residues was determined assuming that the isotope abundance of residual N in the feed was not altered by microbial action. Thus, this content may be derived from the following two equations:

$$\text{N in residue} = \text{feed residual N} + \text{SAB N}$$

$$^{15}\text{N in residue} = \text{feed residual N} \times (^{15}\text{N abundance in residue}) + \text{SAB N} \times (^{15}\text{N abundance in SAB}).$$

Considering also that ^{15}N in residue = N in residue × (^{15}N abundance in residue), the microbial N content can be calculated as follows:

$$\text{Microbial N}(\%) = \frac{^{15}\text{N abundance in residue} - ^{15}\text{N abundance in feed}}{^{15}\text{N abundance in SAB} - ^{15}\text{N abundance in feed}} \times 100.$$

The microbial DM content can be calculated as:

$$\text{Microbial DM}\% = \text{microbial N}\% \times (\%\text{N in residue}/\%\text{N in SAB}).$$

The abundance of ^{15}N in tested feeds for these calculations was determined on the 0 h incubation residues because only the insoluble fraction is subjected to microbial contamination.

The evolution with rumen incubation time (t) of the microbial contamination of the bag residues (M), expressed in terms of DM (mg bacterial DM/100 mg residual DM) and CP (mg bacterial CP/100 mg residual CP), was described for each animal by an exponential model of the first order ($j = 1$) or order different from 1 ($j \neq 1$):

$$\text{M} = m(1 - \text{e}^{-ft})^j.$$

A visual inspection of these results showed that, in most feeds, contamination fitted well to single exponential curves and that only some feeds (CG, CGF, SBP, SBH) showed

Table 1. Chemical composition (g/kg DM) of tested feeds

Feed	Ash	EE	CP	NDF	ADF	ADL	NDIN*	ADIN*
Barley grain	30·5	19·6	128	234	67·9	25·3	10·6	1·44
Maize grain	13·2	31·9	91·2	142	30·0	20·4	5·67	5·27
Sunflower meal	75·2	12·9	398	330	261	97·6	5·13	2·27
Soyabean meal	72·3	10·4	551	89·2	56·7	9·30	2·02	0·43
Full-fat soyabean	56·1	207	408	125	69·9	15·6	2·05	1·03
Maize gluten feed	71·6	15·3	221	463	121	30·1	30·2	6·97
Soyabean hulls	46·4	10·9	105	716	549	26·5	42·4	7·09
Brewers dried grains	40·3	69·3	255	629	267	90·2	17·7	15·7
Sugarbeet pulp	46·4	8·20	110	491	260	42·7	65·2	10·3
Wheat bran	52·4	40·6	182	436	134	48·1	20·5	1·33
Lucerne hay	137	27·0	243	391	305	70·9	3·81	2·97
Vetch-oat hay	150	31·9	144	451	282	44·7	10·9	2·14
Barley straw	97·7	14·0	44·7	750	484	59·7	27·4	6·24
Lentil straw	89·2	9·60	118	599	440	114	13·7	6·37

EE, ether extract; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin.
* Insoluble N in NDF and ADF solutions, respectively (% of total N).
For details of procedures, see this page.

a sigmoid trend. In this last case, the value of j was determined in the regression fits.

In most feeds, the evolution with time (t) of the disappearance (p) of DM or CP (including 0 h values) was described for each animal using the model proposed by Ørskov & McDonald (1979):

$$p = a + b(1 - e^{-kdt}).$$

The disappearance of DM and CP in lucerne hay and barley straw, as well as of CP in lentil straw and in SBP, showed a sigmoid shape and was described using the logistic model of growth of France & Thornley (1984), which is also well adapted to describing rumen degradation:

$$p = a(a + b)/(a + be^{-kdt}).$$

For both models, the constant a represents the soluble or very rapidly degradable fraction, whereas b represents the insoluble degradable component. The undegradable fraction (r) was estimated as $1 - (a + b)$. In the model of Ørskov & McDonald (1979), k_d represents the constant fractional degradation rate of the b fraction. Conversely, in the model of France & Thornley (1984), k_d does not represent the fractional rate of degradation, which is, on the contrary, not constant through the time.

The ED of DM and CP was estimated by using the afore-mentioned equations and the rumen particulate outflow rate (k_p; see later) according to the integration method proposed by Ørskov & McDonald (1979). Thus, when degradation was described with the exponential model, ED was calculated as: $ED = a + (b \times k_d/(k_d + k_p))$. The application of this method to the logistic equation of France & Thornley (1984) leads to the equation:

$$ED = k_p \int_0^\infty [a(a + b)/(a + be^{-k_d t})]e^{-k_p t}\mathrm{d}t.$$

The primitive function of this integral has not been obtained, and therefore ED values were determined by mathematical approximation using a mathematical calculation software (Derive 2; Soft Warehouse Inc., Honolulu, USA).

The rumen fractional outflow rate (k_p) needed to calculate the ED was determined for the vetch-oat hay and the concentrate included in the diet, previously marked by immersion techniques with europium and ytterbium, respectively. Values of k_p for the concentrate were applied to estimate the ED of all concentrates and industrial byproducts, whereas those of vetch-oat hay were applied only to forages. These values were 3·61 (SD 0·192) and 3·29 (SD 0·056), respectively (Rodríguez *et al.* 2000).

Analytical

The chemical composition of the tested feeds was determined using the methods described by the Association of Official Analytical Chemists (1990), except for NDF (Van Soest *et al.* 1991), ADF and acid detergent lignin (Robertson & Van Soest, 1981). The CP concentration of feed and incubated residues was determined as Kjeldahl-N × 6·25. The proportions of insoluble N in NDF and in ADF were also determined by Kjeldahl analysis of the NDF and ADF residues, respectively. Approximate contents of cellulose and hemicelluloses were determined by the differences ADF–acid detergent lignin and NDF–ADF, respectively. The isotope (^{15}N) abundance in samples was determined by MS (VG Prism II IRMS; VG Isotech, Cheshire, UK; linked in series to a Dumas-style N analyser EA 1108; Carlo Erba Instrumentazione, Milan, Italy).

Statistical analysis

All the statistical analyses were performed with the Statistical Analysis System for Windows software, version 6.12 (SAS Institute Inc., Cary, NC, USA). Values of ^{15}N abundance in the whole feed and in its insoluble fraction were compared using the paired Student's t test. Evolutions with time of the contamination (carried out with all the data from each feed) as well as of the apparent and corrected disappearance for DM and CP (carried out by animal and feed) were fitted by non-linear regression. Mean values of apparent and corrected degradation results were compared by variance analysis considering animals as blocks. Predictive equations of the m value of contamination (DM, CP) and of the associated underestimate error of the ED of CP from chemical composition and degradation results of feeds were developed by stepwise multiple linear regression.

Results

There were numerous (eleven) but not systematic differences in the natural abundance of ^{15}N between the whole feed and its insoluble fraction (Table 2). These could be of relevance (up to 0·87 % in SBP) in some feeds.

The evolution with time of the bacterial proportion of the incubated residues as well as the resultant fitted curves are shown in terms of DM in Fig. 1 and in terms of CP in Fig. 2. In SBH, no curves were fitted because the sampling schedule did not allow definition of the asymptotic values. Microbial contamination reached high values in SBP, SBH and all forages. Conversely, these values were low or moderate in wheat bran, brewers dried grains and concentrates, except in CG, which was in the range of forages. Contribution of bacteria to the residues was higher when expressed as mg CP/100 mg total CP, except for SBM, as the CP concentration of residues of this feed was greater than in adherent bacteria.

The kinetics of contamination fitted well to a first-order exponential model, except for CG, CGF and SBP, which showed a sigmoid trend and were fitted with an exponential model of an order different from 1. This same trend may be also considered for SBH (Figs 1 and 2), in which the contamination progressed slowly until 24 h, followed by a strong increment with an hyperbolic shape until 72 h, with values of 22·3 mg bacterial DM/100 mg DM and 72·3 mg bacterial CP/100 mg CP. Also, in SBM, high values were seen at 48 h compared with the exponential fit defined by the data set. These residues represented only 1 % of incubated DM and included a high proportion of hulls (microscopic observation). Consequently, values for this incubation time were not considered to fit contamination curves in this feed. Coefficients of determination (R^2) for these fits varied from 0·850 to 0·971 for the proportion of bacterial biomass and from 0·828 to 0·966 for the contamination with bacterial CP,

Table 2. Natural abundance of ^{15}N (atoms%) in whole feed and its water-insoluble fraction
(Values are means with standard error of the differences)

Feed	Whole feed	Insoluble fraction	SED ($\times 10^{-4}$)	*P*	Difference (%)
Barley grain	0·36 953	0·37 176	0·44	<0·001	0·60
Maize grain	0·36 859	0·37 161	1·81	0·004	0·82
Sunflower meal	0·36 836	0·37 111	0·59	<0·001	0·75
Soyabean meal	0·36 711	0·36 956	0·62	<0·001	0·67
Full-fat soyabean	0·36 781	0·37 064	0·56	<0·001	0·77
Maize gluten feed	0·36 784	0·36 561	3·00	0·085	−0·61
Soyabean hulls	0·36 687	0·36 463	1·56	0·005	−0·61
Brewers dried grains	0·36 774	0·36 606	0·45	<0·001	−0·46
Sugarbeet pulp	0·36 866	0·37 187	0·95	<0·001	0·87
Wheat bran	0·36 714	0·36 700	3·06	0·692	−0·04
Lucerne hay	0·36 747	0·36 727	1·28	0·253	−0·06
Vetch-oat hay	0·36 811	0·36 647	2·23	0·018	−0·45
Barley straw	0·36 892	0·36 950	5·20	0·378	0·16
Lentil straw	0·36 678	0·36 941	0·74	<0·001	0·72

corresponding in both cases to the worst fittings to the CGF sample. The maximum extent of contamination, estimated by the *m* value, varied between 2·84 % and 13·3 % for DM and between 2·85 % and 80·9 % for CP, with maximum values corresponding to barley straw.

Apparent and corrected mean values for the *a*, *b* and *r* fractions, k_d and ED are presented in Tables 3 and 4 for DM and CP, respectively. The apparent degradation kinetics for CP of barley straw were not obtained as a progressive accumulation of CP took place in the bags instead of a disappearance. The restriction $a + b = 100$ (values expressed as a percentage) was needed for both DM and CP in all animals for SBM and full-fat soyabean, as well as in some animals for the apparent disappearance values of DM (SBH, CG) and of CP (CG, CGF, SBP).

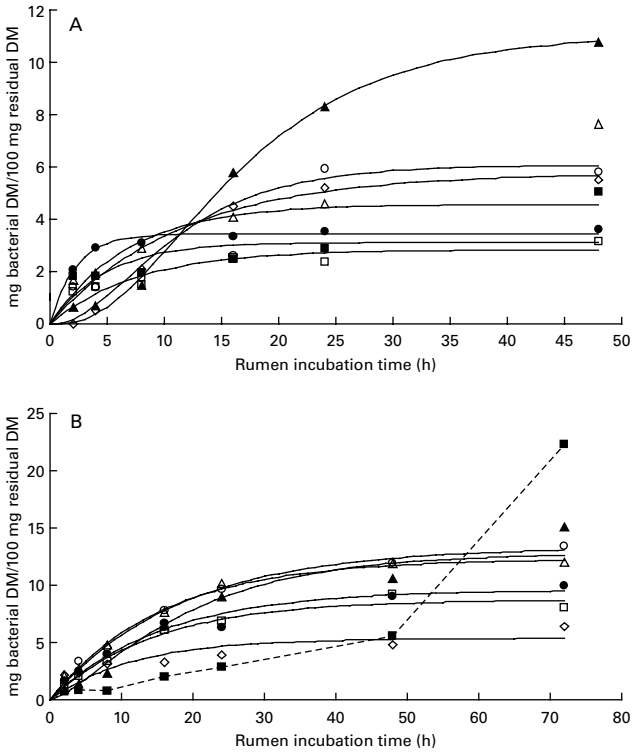

Fig. 1. Accumulation of biomass (mg bacterial DM/100 mg residual DM) during rumen incubation and resulting fitting curves of (A) concentrates and industrial byproducts (wheat bran (□), barley grain (■), full-fat soyabean (○), sunflower meal (●), maize grain (▲), maize gluten feed (◇), soyabean meal (Δ)) or (B) forages and cell wall-rich byproducts (lentil straw (□), barley straw (○), lucerne hay (●), sugarbeet pulp (▲), brewers dried grains (◇), vetch-oat hay (Δ), soyabean hulls (■)). Symbols are mean values for four animals and two bags per animal. For details of procedures, see p. 317.

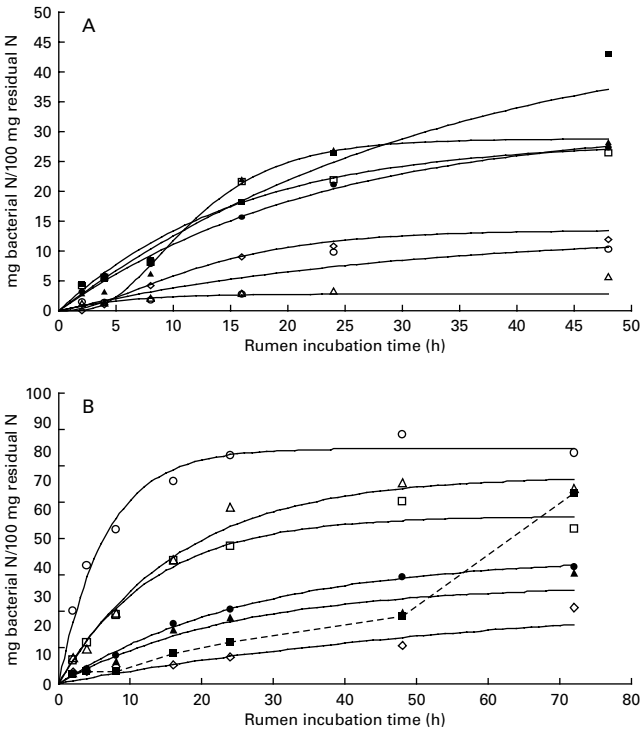

Fig. 2. Evolution of microbial contamination (mg bacterial N/100 mg residual N) during rumen incubation and resulting fitting curves of (A) concentrates and industrial by-products (wheat bran (□), barley grain (■), full-fat soyabean (○), sunflower meal (●), maize grain (▲), maize gluten feed (◇), soyabean meal (Δ)) or (B) forages and cell wall-rich byproducts (lentil straw (□), barley straw (○), lucerne hay (●), sugarbeet pulp (▲), brewers dried grains (◇), vetch-oat hay (Δ), soyabean hulls (■)). Symbols are mean values for four animals and two bags per animal. For details of procedures, see p. 317.

Table 3. Effect of correction (Cor) for microbial contamination on mean degradation kinetics parameters and effective degradability (ED) of DM
(Values are means for four animals with standard error of the differences)

Item		BG	CG	SFM	SBM	FSB	CGF	SBH	BDG	SBP	WB	LH	VH	BS	LS
a	App	34·1	18·2	27·1	32·9	41·1	28·4	11·7	10·6	9·38	42·9	30·8	38·7	14·2	28·2
	Cor	34·1	17·8	27·2	33·3	41·7	28·1	11·8	11·5	9·36	42·9	31·3	38·9	15·2	28·2
	SED	0·004	0·065	0·015	0·044	0·037	0·105	0·200	0·077	0·061	0·011	0·015	0·081	0·196	0·127
	P	0·097	0·009	0·017	0·001	<0·001	0·061	0·836	0·001	0·924	0·005	<0·001	0·037	0·012	0·877
b	App	53·2	77·2	46·6	67·1	58·8	61·8	72·6	57·3	86·4	32·9	45·1	41·1	37·3	34·3
	Cor	53·5	76·8	47·3	66·6	58·2	62·3	77·1	57·5	86·7	33·4	46·8	43·1	41·0	37·4
	SED	0·047	1·114	0·097	0·044	0·037	0·364	2·353	0·290	0·599	0·027	0·431	0·100	0·483	0·128
	P	0·008	0·721	0·007	0·001	<0·001	0·258	0·153	0·532	0·661	<0·001	0·029	<0·001	0·004	<0·001
r	App	12·7	4·59	26·3	0·00	0·00	9·82	15·6	32·1	4·24	24·2	24·0	20·2	48·5	37·5
	Cor	12·4	5·41	25·5	0·00	0·00	9·61	11·1	31·0	3·95	23·6	21·9	17·9	43·8	34·4
	SED	0·046	1·174	0·110	NA	NA	0·337	2·446	0·220	0·571	0·031	0·424	0·158	0·419	0·165
	P	0·008	0·534	0·007	NA	NA	0·595	0·161	0·015	0·652	<0·001	0·014	<0·001	0·001	<0·001
k_d (%/h)	App	57·3	5·56	17·7	7·92	7·18	6·24	2·73	3·80	4·49	13·8	12·5	5·69	8·10	5·72
	Cor	58·2	6·08	18·8	8·17	7·36	6·66	2·73	4·07	4·86	14·2	13·1	6·23	9·40	6·27
	SED	0·235	0·138	0·073	0·028	0·019	0·113	0·094	0·024	0·048	0·081	0·120	0·101	0·322	0·053
	P	0·029	0·033	<0·001	0·003	0·002	0·035	0·971	0·001	0·004	0·018	0·014	0·013	0·027	0·001
ED (%)*	App	84·1	64·8	65·8	78·7	80·2	67·2	42·3	39·7	56·9	68·9	62·4	64·7	34·3	49·9
	Cor	84·4	65·9	66·8	79·2	80·8	68·2	44·4	41·7	58·9	69·5	64·4	67·1	39·1	52·6
	SED	0·049	0·211	0·084	0·067	0·021	0·153	0·504	0·084	0·148	0·024	0·194	0·139	0·448	0·184
	P	0·007	0·015	0·001	0·003	<0·001	0·008	0·027	<0·001	<0·001	<0·001	0·001	<0·001	0·001	<0·001

BG, barley grain; CG, maize grain; SFM, sunflower meal; SBM, soyabean meal; FSB, full-fat soyabean; CGF, maize gluten feed; SBH, soyabean hulls; BDG, brewers dried grains; SBP, sugarbeet pulp; WB, wheat bran; LH, lucerne hay; VH, vetch-oat hay; BS, barley straw; LS, lentil straw; *a*, *b*, *r*, soluble, non-soluble degradable and undegradable fractions, respectively; k_d, fractional degradation rate; App, apparent; NA, non-analysed.
*Calculated with rumen outflow rates (%/h) of 3·61 (SD 0·192) in concentrates and industrial byproducts and 3·29 (SD 0·056) in forages.
For details of procedures, see p. 317.

Table 4. Effect of correction (Cor) for microbial contamination on mean degradation kinetics parameters and effective degradability (ED) of crude protein

(Values are means for four animals with standard error of the differences)

Item		BG	CG	SFM	SBM	FSB	CGF	SBH	BDG	SBP	WB	LH	VH	BS	LS
a	App	34·3	19·2	30·9	20·0	37·4	36·1	22·6	10·5	16·1	46·5	39·7	55·1	NA	63·6
	Cor	34·5	18·1	30·9	20·4	38·0	35·8	24·3	11·8	18·8	46·7	40·9	55·6	50·2	64·1
	SED	0·040	0·313	0·005	0·020	0·035	0·189	0·343	0·186	0·509	0·022	0·388	0·195	NA	0·478
	P	0·026	0·043	0·017	<0·001	<0·001	0·226	0·014	0·006	0·014	0·005	0·055	0·080	NA	0·435
b	App	60·5	78·4	63·6	79·9	62·5	61·3	43·8	76·6	73·7	46·2	50·2	33·8	NA	15·5
	Cor	61·6	75·1	64·5	79·6	61·9	61·1	63·0	77·5	71·8	47·7	53·1	41·2	36·8	26·8
	SED	0·161	1·983	0·126	0·036	0·035	0·633	4·357	0·328	4·534	0·063	0·897	0·360	NA	0·536
	P	0·007	0·199	0·005	0·002	<0·001	0·688	0·021	0·071	0·702	<0·001	0·050	<0·001	NA	<0·001
r	App	5·06	2·46	5·48	0·00	0·00	2·59	33·6	12·9	10·1	7·25	10·1	11·1	NA	20·8
	Cor	3·84	6·77	4·56	0·00	0·00	3·16	12·6	10·7	9·40	5·61	6·05	3·20	13·0	9·12
	SED	0·179	2·103	0·128	NA	NA	0·616	4·683	0·409	4·205	0·070	0·733	0·503	NA	0·560
	P	0·006	0·132	0·005	NA	NA	0·425	0·020	0·012	0·875	<0·001	0·011	<0·001	NA	<0·001
k_d (%/h)	App	28·2	3·40	25·9	8·10	7·92	4·16	5·65	4·18	7·27	20·7	12·8	7·95	NA	6·04
	Cor	29·0	5·35	27·1	8·23	8·14	4·85	3·99	4·54	8·29	21·5	13·6	9·78	12·4	9·81
	SED	0·296	0·161	0·048	0·020	0·030	0·182	0·671	0·034	0·390	0·239	0·256	0·373	NA	0·260
	P	0·062	0·001	<0·001	0·006	0·005	0·031	0·089	0·001	0·079	0·054	0·051	0·016	NA	<0·001
ED (%)*	App	88·0	57·0	86·6	74·6	80·3	68·6	48·9	50·8	46·5	85·8	75·4	78·9	NA	73·0
	Cor	89·2	62·9	87·7	75·0	80·8	70·5	56·2	54·3	53·5	87·4	79·2	86·4	77·0	83·0
	SED	0·172	0·450	0·105	0·043	0·025	0·312	1·043	0·191	0·809	0·068	0·341	0·528	NA	0·631
	P	0·005	<0·001	0·001	0·002	<0·001	0·009	0·006	<0·001	0·003	<0·001	0·001	<0·001	NA	<0·001

BG, barley grain; CG, corn grain; SFM, sunflower meal; SBM, soyabean meal; FSB, full-fat soyabean; CGF, corn gluten feed; SBH, soyabean hulls; BDG, brewers dried grains; SBP, sugarbeet pulp; WB, wheat bran; LH, lucerne hay; VH, vetch-oat hay; BS, barley straw; LS, lentil straw; *a*, *b*, *r*, soluble, non-soluble degradable and undegradable fractions, respectively; k_d, fractional degradation rate; App, apparent; NA, non-analysed.

*Calculated with rumen outflow rates (%/h) of 3·61 (SD 0·192) in concentrates and industrial byproducts and 3·29 (SD 0·056) in forages.

For details of procedures, see p. 317.

The correction for the microbial contamination (Tables 3 and 4) had little effect on estimates of the soluble fraction, whereas this correction led to significant increases in the *b* fraction in most feeds. Conversely, significant but low reductions in this fraction for both DM and CP were observed in SBM and full-fat soyabean. Reductions of the estimates of the *r* fraction of both DM and CP in most feeds were also observed, except for CG, CGF and SBP. Finally, microbial correction led to an increase in k_d estimates, although this effect was only slightly significant ($P<0{\cdot}1$) for CP in barley grain, SBP, wheat bran and lucerne hay. The only exceptions were the k_d rates (DM, CP) of SBH, for which no effect was appreciated.

The correction of microbial contamination led, in all the tested feeds, to significant increases in the estimates of ED (Tables 3 and 4). The undervaluation of ED was in general lower for DM than for CP. For the latter, the importance of the error was low for the concentrates, except for CG, in which the underestimate reached 9·49 %, whereas in the fibrous byproducts and forages this error varied between moderate and high. The associated overestimation errors for the rumen undegraded protein were amplified with the rise in the corrected ED of CP, and could therefore vary greatly between feeds. This proportion was low only in SBM (1·74 %) and full-fat soyabean (2·84 %), moderate in the other protein concentrates (CGF 6·00 %, sunflower meal 8·35 %) and in brewers dried grains (7·06 %), high in cereals (barley grain 10·6 %, CG 13·9 %) and in the other industrial byproducts (wheat bran 11·4 %, SBP 13·1 %, SBH 14·1 %), and very high in forages (vetch-oat hay 35·4 %, lentil straw 36·9 %), except in lucerne hay (15·6 %).

Equations predicting the *m* values of contamination from the chemical composition and degradability of feeds are shown in Table 5. The CG sample was excluded from this study for reasons discussed later. The main factor affecting both DM and CP contamination was the feed cellulose concentration (R^2 0·623 and 0·646, respectively). For DM, negative effects were recorded for the contents of apparent undegradable DM and acid detergent lignin (accumulated R^2 0·919 and 0·949, respectively). Finally, a positive effect of the hemicellulose content was observed (accumulated R^2 0·964). For CP, negative effects were observed for the CP content and the insoluble N in NDF solution of the feed (accumulated R^2 0·773 and 0·898, respectively).

The underestimation error of ED of CP (Table 5) was also correlated with chemical parameters, as well as with the apparent values of the undegradable fraction and ED of both DM and CP. CG was also not included in these studies. The main independent variable was the cellulose content, with a coefficient of determination (R^2) of 0·702. Two additional independent variables were negatively related: the apparent ED of CP (accumulated R^2 0·789) and the CP content (accumulated R^2 0·867).

Discussion

Contamination of feed particles

Differences in the ^{15}N:N ratio shown in Table 2 demonstrate that the ^{15}N distribution between CP fractions was not strictly uniform. These differences may lead to large errors in estimates of CP contamination, especially for short incubation times. For example, for SBP (which showed the maximum difference), contamination estimates from the whole-feed ^{15}N:N ratio overestimated those from the insoluble fraction by 56·9 %, 7·52 %, 6·67 % and 3·23 % for 2, 16, 48 and 72 h of incubation, respectively. In CGF, in which there was an inverse effect, the use of the first-cited ratio value led to a negative and therefore non-biological result at 2 h incubation (− 1·35 % *v.* 0·20 %). This underestimation was also important at other incubation times (e.g. 14·4 % and 10·6 % for 16 and 48 h, respectively). Therefore, the ^{15}N:N value employed for calculations is an important source of variation in these studies and should be derived from the insoluble feed fraction as only this fraction is subjected to microbial contamination.

The present results show that microbial contamination in terms of DM or N of all feeds follows a curvilinear increase with rumen residence time, supporting the results of Nocek (1988), who indicated that bacteria continually attach to particles to saturate all

Table 5. Prediction equations for maximum (asymptotic *m* value) contamination (mg bacterial DM or crude protein (CP)/100 mg residual DM or CP) and associated underestimation error of the corrected CP effective degradability (*n* 12)

	Residual SD	R^2	*P*
DM contamination*			
[1] 2·84 (± 1·31) + 0·25 (± 0·06)C†	2·49	0·623	0·002
[2] 3·40 (± 0·65) + 0·47 (± 0·05)C − 0·22 (± 0·04)DM*u*‡	1·22	0·919	< 0·001
[3] 4·03 (± 0·61) + 0·47 (± 0·04)C − 0·17 (± 0·04)DM*u* − 0·31 (± 0·14)ADL†	1·02	0·949	< 0·001
[4] 3·25 (± 0·71) + 0·49 (± 0·04)C − 0·20 (± 0·04)DM*u* − 0·26 (± 0·13)ADL + 0·05 (± 0·03)HC†	0·91	0·964	< 0·001
CP contamination*			
[5] 10·2 (± 7·78) + 1·58 (± 0·37)C†	14·7	0·646	0·002
[6] 36·2 (± 13·3) + 1·05 (± 0·39)C − 0·71 (± 0·31)CP†	12·4	0·773	0·001
[7] 57·4 (± 11·6) + 0·96 (± 0·28)C − 1·12 (± 0·26)CP − 0·56 (± 0·18)NDIN§	8·86	0·898	< 0·001
Underestimation error (%) of the corrected ED of CP‖			
[8] 0·11 (± 1·38) + 0·29 (± 0·06) C†	2·82	0·702	< 0·001
[9] 9·81 (± 5·21) + 0·22 (± 0·06) C − 0·12 (± 0·06) CP ED‡	2·50	0·789	< 0·001
[10] 13·5 (± 4·69) + 0·16 (± 0·06) C − 0·11 (± 0·05) CP ED − 0·11 (± 0·05) CP†	2·10	0·867	< 0·001

C, cellulose (acid detergent fibre minus acid detergent lignin); DMu, apparent undegradable DM; ADL, acid detergent lignin; HC, hemicelluloses; NDIN, insoluble N in neutral detergent fibre; ED, effect degradability.

* Corn grain excluded, soyabean hulls not determined.

† % on DM.

‡ %,xxx.

§ % on total N.

‖ Corn grain excluded, barley straw not determined.

the attachment points. This evolution can be fitted with simple models to simplify the interpretation of multiple data (González *et al.* 2006). Specifically, the asymptotic value (*m*) is more interesting because its prediction allows a correction of apparent estimates of the undegradable fraction (see later), and it is a good basis on which to discriminate the main factors affecting contamination in the rumen.

The first-order exponential model, observed for most samples, agrees with the exponential supply to microcolonies of nutrients derived from the degradation of their substrates, whereas the sigmoid evolution seen with some feeds (CG, CGF, SBH and, at a lower level, SBP) may be due to an irregular progression of the colonisation, associated with the structure of plant tissues. This same trend can be observed in previous results for CG (Bernard *et al.* 1988, Valadares Filho *et al.* 1992). The vitreous endosperm of this feed is composed of a matrix of horny starch and proteins (prolamines and gluteins), which offers resistance to the hydrolytic actions of microbial enzymes (Rooney & Pflugfelder, 1986). Therefore, this protein matrix is extremely resistant to attachment and penetration (McAllister *et al.* 1993), which makes the progression and development of bacterial microcolonies quite difficult. In addition, the germ of maize is resistant to microbial penetration (McAllister *et al.* 1990) and is therefore a durable substrate for microcolonies. In accordance with the microscopic observations of Grenet & Barry (1987), SBH includes tissues of very fast desegregation, such as the parenchyma (which avoids the initial development and accumulation of bacterial microcolonies), or tissues that are moderately or extremely resistant to degradation (subepidermic layer and epidermis, respectively), which should lie at the origin of the marked sigmoid evolution. A similar schema may be considered for CGF, which is composed of different byproducts with different degrees of resistance to degradation. Thus, part of the bacterial microcolonies would be eliminated by the fast desegregation of the floury starch, whereas the fibrous maize fractions, the maize germ and the rest of the vitreous endosperm might allow a durable development of these microcolonies.

The use of an exponential model with an order different from 1 in these feeds leads to higher R^2 values than are seen with the simple model and avoids overestimating *m* values in relation to the last incubation time (assumed to be close to *m* values). Thus, for DM contamination, R^2 values were 0·850 *v.* 0·838 for CG and 0·944 *v.* 0·928, respectively, for CGF, whereas *m* values were 11·1 % and 15·6 % *v.* 11·0 % at 48 h for CG and 6·09 % and 6·93 % *v.* 6·18 % at 48 h for CGF, respectively. For SBP, all these differences were small for both models.

Overall, previous results (Varvikko & Lindberg, 1985; Bernard *et al.* 1988; Ould-Bah *et al.* 1988; Valadares Filho *et al.* 1992; Wanderley *et al.* 1993; Zakraoui, 1996) and the present findings demonstrate low or moderate values of contamination with N in concentrates, except in CG, and high contamination levels in forages or byproducts rich in fibre. In forages, the present results also support a higher contamination in grass than in legumes, as previously observed (Nocek & Grant, 1987, Bernard *et al.* 1988).

Rumen degradation

The need to assume a value of zero for the undegradable fraction is frequent in feeds with a low value for this fraction, such as SBM and full-fat soyabean. The progressive enrichment of CP in barley straw residues is common in feeds with high fibre and low CP contents (Varvikko & Limberg, 1985; Varvikko, 1986).

The effects of subtracting the microbial contamination of rumen-incubated residues on the kinetic degradation parameters were those mathematically predictable: a reduction in the *r* fraction associated with an increase in the *b* fraction and in its degradation rate. Initially, the *a* fraction should not be affected. However, this estimate was obtained in the present experiment by the intersection of this kinetic with the ordinate axis, and may therefore be affected by the changes indicated earlier. The exceptions to this general outline were always derived from mathematical interferences related to the indicated restriction for the *r* value. Thus, when this practice was only necessary for the apparent fit, this value was underestimated, which could also have had an effect on other degradation parameters. Also, when *r* was always assumed to be zero (SBM, full-fat soyabean), the effect of the correction could only be translated into an increase and an equivalent reduction in the *a* and *b* fractions, respectively, as observed by Beckers *et al.* (1995). Therefore, the effects associated with this correction can be expressed in different ways in terms of the limitations of the models.

The final effect of all these changes was an increase in ED values, in agreement with previous results (Mathers & Aitchison, 1981; Rooke *et al.* 1984; Bernard *et al.* 1988; Ould-Bah *et al.* 1988; Valadares Filho *et al.* 1992; Kamoun *et al.* 1993; Beckers *et al.* 1995; González *et al.* 2006).

Prediction of microbial contamination and the associated error on degradability estimates of crude protein

The possibilities of a long-term development of bacterial microcolonies on feed particles depends on their content of materials that are relatively resistant to ruminal degradation. In feeds of vegetable origin, these characteristics are mainly represented by the cell walls and by the horny starch. Among the samples tested, only CG was rich in horny starch, whereas its cell wall content was very low. Because of the difference in factors that determine the permanency of microcolonies, this feed was excluded *a priori* from the regression analyses.

The equations obtained showed that cellulose was the structural carbohydrate responsible for the level of development of microcolonies, whereas the content in hemicelluloses did not have a large influence as its coefficient in equation [4] (Table 5) was ten times lower than that of cellulose. This limited role may be explained by their associations with the lignin through covalent links, which allows a lower colonisation by the micro-organisms (Hatfield, 1993; Van Soest, 1994), and also through bridges of phenolic acids, which have a slight toxicity for the cellulolytic bacteria (Chesson *et al.* 1982). The high colonisation of SBP (with similar contents of cellulose and hemicelluloses) seems to indicate a higher contribution of hemicelluloses to the development of microcolonies on low-lignified cell walls.

The negative effect of the acid detergent lignin concentration on microbial accumulation agrees with microscopic observations indicating a low colonisation of lignified tissues (Akin, 1979; Harbers *et al.* 1981, Cheng *et al.* 1984). In addition, Chesson & Forsberg (1988) indicated that when

microcolonies reach the cell wall's lignified layers, these layers prevent or limit degradation. The negative effect of the proportion of apparent undegradable DM in the feed may translate the effects of other materials that it is difficult to colonise or that limit the progression of the degradation, i.e. other phenolic compounds (hidroxicinamic acids, polyphenols, tannins) or the cutin, silica and waxes of the epidermis (McAllister *et al.* 1994). On the other hand, the effects of lignin do not depend exclusively on its concentration in the feed, as its distribution in cell walls and in vegetable tissues determines its effectiveness in preventing microbial attack (Grenet & Demarquilly, 1987), which may be reflected in the values for undegradable DM.

As cellulose content was the main factor determining the accumulation of microcolonies on feed particles, it was also responsible for the accumulation of microbial N. The increase in CP in the feed concentration has a diluting effect on the contribution of microbial CP to the residue. Similarly, the increase in insoluble N in the NDF solution slows down the degradation of CP (Pereira *et al.* 1998; Alvir *et al.* 1999; González *et al.* 1999; Haj Ayed *et al.* 2000) and therefore has the same effect.

The two former variables were also identified (for the reasons indicated before) as main variables affecting the underestimation of the ED of CP. The dilution effect of a higher feed CP content has already been indicated (Mathers & Aitchison, 1981; Michalet-Doreau & Ould-Bath, 1989; Wanderley *et al.* 1993). In addition, this error is also diminished with the increase in apparent ED of CP as effects of contamination are expressed more intensely when the feed has not been degraded. However, a mathematical effect associated with the expression as a percentage of this error should be also considered, as the relationship was worse when it was expressed as a difference (corrected minus apparent values) and was based only on the contents of cellulose and CP.

Equation [10] (Table 5) shows that the error in ED of CP derived from microbial contamination depended on the feed chemical and degradative characteristics and might therefore vary widely between different batches of the same feed; thus, the data for this error reviewed by González *et al.* (2006) in lucerne hay (which ranged between 4·85 % and 14·1 %) were closely correlated with the feed CP content (r 0·96), as well as with its apparent ED (r 0·94). This equation should not be applied to grains rich in horny starch, such as maize or sorghum (the inclusion of CG in the data base reduced the R^2 of equation [8] (Table 5) from 0·702 to 0·477), which could be studied in more detail.

As indicated in Results, the microbial contamination was also associated with an overestimation of the undegraded protein in the feed. These errors enhance those in the ED of CP. Therefore, this microbial fraction can induce an additional error in the *in situ* estimates of the intestinal digestibility of CP. The microbial contamination of feeds in the rumen also had implications for the accuracy of current rationing protein systems using *in situ* methodologies, owing to a partial duplication (variable with the feed) in the estimation of the microbial CP supplied to the duodenum.

Conclusion

In situ studies of feed microbial contamination may be improved by fitting the data obtained at the different rumen incubation times to exponential models. Also, to increase accuracy, the ^{15}N:N value should be determined on the insoluble feed fraction instead of on whole feed.

Except for feeds rich in horny endosperm (i.e. maize), cellulose is shown as the main substrate responsible for microbial DM accumulation and its associated CP contamination. The equation derived from this parameter together with other feed characteristics (the CP content and its apparent ED) allowed us to predict the underestimation of the ED of CP, and therefore provide a better evaluation of feed protein.

Overestimation errors of the rumen undegraded protein values as a result of such microbial contamination may be important in many feed types, especially in forages and fibre byproducts. The microbial fraction included in the undegraded residues may also cause errors in estimates of the intestinal digestibility of CP. This correction is therefore needed at the ruminal and intestinal levels to obtain reliable feed protein values.

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