Effects of correcting *in situ* ruminal microbial colonization of feed particles on the relationship between ruminally undegraded and intestinally digested crude protein in concentrate feeds

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Running title: effect of rumen-microbial colonization on intestine-digested protein prediction

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Abstract

BACKGROUND: *In situ* estimates of ruminally undegraded protein (RUP) and intestinally digested protein (IDP) of ten concentrates, uncorrected or corrected for the ruminal microbial colonization, were used to examine the effects of this correction on the relationship between IDP and RUP values. Both variables were established for three rumen and duodenum cannulated wethers using ^{15}N labeling-techniques and considering measured rates of ruminal particle comminution (k_c) and outflow (k_p).

RESULTS: A covariance analysis showed that the close relationship found between both variables ($\text{IDP} = -0.0132 (\pm 0.00679) + 0.776 (\pm 0.0002) \text{RUP}$; $n = 60$; $P < 0.001$; $R^2 = 0.960$) is not affected by correcting for microbial colonization ($P = 0.682$).

CONCLUSION: The IDP content in concentrates and industrial by-products can be predicted from RUP values, thus avoiding the laborious and complex procedure of determining intestinal digestibility; however a larger sample of feeds is necessary to attain more accurate predictions. The lack of influence of the correction for microbial contamination on the prediction observed in our study increases the data available for this prediction. However, only using corrected values may provide an accurate evaluation.

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1. Introduction

The feed protein evaluation in most current rationing systems for ruminants considers the supply of ruminally degraded and undegraded protein, the synthesis of microbial protein in the rumen and the intestinal digestion of the post-ruminal flow of protein. For dietary protein, the rate and extent of ruminal degradation are not only major determinants of this protein flow, but also of its composition and, therefore, of its intestinal digestion^{1,2}. The *in situ* methods are suitable to perform systematic measures of ruminal and intestinal digestion of multiple feeds; therefore, their results, and especially those of ruminal degradability, are used in numerous feed rationing systems such as NRC³, INRA⁴, DVE/OEB⁵ and Norfor⁶. However, these methods may present some accuracy problems, such as the colonization of ruminal undigested residues with adherent microorganisms, which will be largely digested during the intestinal incubations. As a consequence, both estimates (ruminal degradability and intestinal digestibility) are biased. In addition, as ruminal degradation determines the digestion site, any error made in this determination leads to another in the intestinal digestibility estimates. Therefore, it is advisable to consider the total rumen residence time of feed particles as determined by their outflow rate from the reticulum (k_p) as well as by their rate of comminution and mixing (k_c)^{7,8}, although the latter rate is often not considered in *in situ* studies. Based on the previously indicated interrelation between ruminal degradation characteristics and intestinal digestion, the objective of this work was to investigate the possible relationship between the contents of intestinally digested crude protein (IDP) and ruminally non-degraded protein (RUP) of concentrate feeds and to evaluate the effects of correcting the microbial colonization taking place in the rumen on this relationship.

2. Materials and methods

2.1. Experimental

Ten samples of concentrates and industrial by-products of the food industry were tested in one experiment on three wethers fitted with rumen and T-type duodenal cannula and fed a ration with a 2:1 ratio (fresh matter basis) of chopped oat hay to commercial

concentrate. The chemical composition of the tested feeds as well as that of oat hay and concentrate is shown in Table 1. This diet was fed 10 days before the onset of the experimental period, at 40 g kg^{-1} body weight^{0.75} divided in six equal meals offered every 4 hours. The animals were handled in accordance with the Spanish guidelines for experimental animal protection, published in the Royal Decree 53/2013⁹. The values for microbial contamination, RUP and IDP measured for different feeds in the animal experiment and used in this study are shown in Table 2.

In situ studies included nylon-bag incubations in rumen and intestine, as well as measurement of the ruminal transit of dietary concentrate particles and the correction of the microbial colonization taking place in the rumen in both types of samples. Ruminal incubations were performed for 2, 4, 8, 16, 24 and 48 h using nylon bags (46 μm of pore size and internal dimension of $110 \times 70 \text{ mm}$). Bags were filled with approximately 3 g (air-dry basis) of each feed previously ground to pass a 2 mm screen. A total of 10 incubations carried out in two periods of five incubations were performed in order to have two bags for each incubation time and feed. In each incubation all bags of the different incubation times (6 for each feed) of two randomly associated feeds were simultaneously placed in the rumen immediately before the first meal in the morning (0900 h). After being collected from the rumen, bags were washed with tap water and stored at -20°C . After thawing, bags were washed three times for 5 minutes in a mini-turbine washing machine (Jata 580; JATA, Abadiano, Bizkaia, Spain) and frozen again (-20°C). Finally, bags were freeze-dried and immediately weighed to establish DM degradation kinetics. Between both periods of ruminal incubations, the rates of particle comminution (k_c) and transit (k_p) in the rumen-reticulum were determined. For this purpose the wethers were pulse-dosed before the first morning meal with 50 g of the dietary concentrate labelled with Ytterbium (using a solution of YbCl_3) and the marker evolution was checked in 21 duodenal samples recovered during the following 96 h. The model of Dhanoa et al.¹⁰ was fitted to the marker concentration data of each animal and primary and secondary rate constants of this model were assumed as k_p and k_c values, respectively, in agreement with Ellis et al.⁷ and González et al.⁸. To account for the above mentioned microbial colonization, a solution of ^{15}N enriched ammonium sulphate (98 atoms%) was continuously infused into the rumen (25 mg ^{15}N per wether and day) during these incubations, up to the isolation of solid adherent bacteria, which were used as reference for these corrections. For this purpose, the rumen was manually

emptied and a representative sample of 1.2 kg was subjected to a double centrifugation procedure, as described by Rodríguez et al. (2000)¹¹. Degradation kinetics of DM were fitted to the single exponential model of Ørskov and McDonald¹², except in some feeds which needed specific models: corn gluten feed, which showed two consecutive single exponential curves², and dehydrated sugar beet pulp, which showed a sigmoid evolution¹³. Estimates of RUP were established by generating in each wether a composite sample representative of the feed by-pass based on the function describing the feed rumen outflow developed by Arroyo and González¹⁴ considering k_c and k_p particle transit rates and DM degradation kinetics. To generate these composite samples, the residues obtained at each incubation time for each animal were pooled in equal quantities. Then, the resultant residues for 0, 2, 4, 8, 16, 24 and 48 h of incubation were considered representative of the composition of the rumen outflow of undegraded feed in the intervals 0–1, 1–3, 3–6, 6–12, 12–20, 20–36 and 36–72 h, respectively. Finally, the rumen outflow of undegraded feed in these intervals was established by the above indicated function of Arroyo and González¹⁴ and the proportions in which the different residues were mixed were calculated by the quotient between the flow in each interval and the total flow. These composite samples were analysed for CP to determine RUP values and also incubated through the intestine in mobile nylon bags to establish intestinal digestibility values. To avoid errors in the correction of the ruminal microbial colonization of the intestinal undigested samples, these incubations were performed after a 10-day resting period from the ruminal study to eliminate the ¹⁵N enrichment in the digesta. Mobile bags were randomly inserted through the duodenal cannula at a rate of one bag every 15 min (8 bags per wether and day), recovered from the faeces and processed as indicated for ruminal incubations. Finally, bags were dried (70°C for 48 h) and weighed to calculate the intestinal effective digestibility (IED) of DM. Then, the residues were pooled for each wether before CP analyses to determine IED of RUP. Additional details on ruminal and intestinal incubations, ruminal transit, ruminal degradation models, solid adherent bacteria isolation, microbial colonization, calculations and analytical procedures have been previously summarised^{2,13,15}. Mean results of k_c and k_p rates, ruminal microbial contamination with crude protein (RMC), RUP, its IED and IDP were also presented in the former articles, as well as in Table 2.

2.2. Chemical analyses

Analyses were performed according to the methods of the Association of Official Analytical Chemists¹⁶ for DM (air-forced oven dehydration; procedure 934.01), ash (incineration in muffle furnace; procedure 967.05), ether extract (ether extraction; procedure 920.39) and CP (Dumas N $\times$ 6.25; procedure 968.06). Feedstuff samples were analysed for NDF¹⁷, using a heat stable α -amylase and without sodium sulphite, and for direct acid detergent fibre (ADF) and acid detergent lignin¹⁸. Both NDF and ADF were expressed inclusive of residual ash. Contents of neutral-detergent insoluble nitrogen (NDIN) and acid-detergent insoluble N (ADIN) were determined by N analysis of the respective fibre residues with the previously cited procedure for N.

2.3. Statistical analyses

A covariance analysis using individual values was done in order to study the linear relationship between IDP and RUP considering the microbial contamination occurred in the rumen (c) as a fixed effect and RUP as linear covariate. The interaction between both variables was also included in the model: $IDP_{ij} = \beta_0 + c_i + \beta_1 RUP_{ij} + \sum_i \beta_{2i} (c \times RUP)_{ij} + \varepsilon_{ij}$, where $\beta_0, \beta_1, \beta_{2i}$ are regression parameters. This analysis was developed with the GLM procedure of SAS (version 9.3)¹⁹.

3. Results and discussion

The covariance analysis showed that the close relationship found between IDP and RUP (Figure 1; $IDP = -0.0132 (\pm 0.006795) + 0.776 (\pm 0.0002) RUP$; $n = 60$; $P < 0.001$; $R^2 = 0.960$) is not affected by either the correction for ruminal microbial colonization or the RUP $\times$ microbial colonization interaction ($P = 0.682$ and 0.991 , respectively). The lack of influence of the correction for microbial colonization indicates similar intersects for both microbial corrected and noncorrected data series, whereas the lack of significance of the interaction shows that both data series have the same regression slope. As a consequence, both noncorrected and corrected values can be used together to perform regression equations to predict IDP. As RUP and IDP data obtained considering effective RUP values are scarce in the literature, the possibility of using both types of values for this prediction is of great interest to attain accurate predictions. Using non-microbial corrected values from 35 samples of concentrates and co-products and 17 samples of forages, González²⁰ showed negative relationships between IDP values and those of CP effective degradability with similar determination coefficients to those of

the present study. This is in agreement with the positive relationship found with RUP, complementary value to that of degradability.

As shown in Table 2, not correcting the microbial colonization taking place in the rumen overestimates both RUP and IED and, therefore, their product (IDP) in sugar beet pulp¹³, rapeseed meal and palm kernel¹⁵. This same behaviour was observed in cereal grains and cereal co-products², although in some feedstuffs of this group only a trend ($P < 0.1$) or just numerical differences (i.e. for IED values) were detected. Current colonization results of RUP are in agreement with the indications of Rodríguez and González²¹, who established that adherent microbial biomass is directly determined by the feed content in slowly degradable fractions such as corn-vitreous starch or fibre (specially cellulose) and inversely affected by the feed contents in undegradable components and lignin. These same authors showed that the contamination with microbial N decreases when the feed N content and its proportion bound to fibre increase. They also showed that the overestimations of RUP due to microbial colonization increase when RUP decreases and even more so when it is expressed as percentage. Thus, values of the microbial fraction in RUP as well as its associated overestimations were low in feeds rich in CP (distillers dried grains with solubles or rapeseed meal) or in fibre bound-N (palm kernel), whereas they were high in feeds with quickly degradable proteins (rye and wheat grains) or in low-protein feeds rich in slowly degradable components (fibre in dehydrated sugar beet pulp and vitreous starch in corn grain). As ruminal microorganisms that are adhered to feed particles are largely digested in the intestine^{2,13,15}, the overestimation of the IED of RUP is not only defined by the magnitude of the ruminal colonization but mainly by the difference between the true intestinal digestibility of nitrogenous compounds from microorganisms and the truly undegraded feed fraction^{2,15}. Thus, the overestimation of IED due to the ruminal microbial contamination in the sample set of Table 2 ranged from 0.7 to 43.7%, being inversely proportional to noncorrected IED values as shown by the correlation coefficient between these parameters (-0.883). In addition, it should be considered that any increase in ruminal degradation of a feed simultaneously increases the proportion of indigestible components in its post-ruminal outflow and thus causes a reduction in its true intestinal digestibility. Therefore, a greater degradation implies an increase in the overestimation of the IED of RUP associated with its microbial colonization. For example, the similar RUP overestimation observed in rye and corn grains (22.1% and

22.5%, respectively) was associated with an overestimation of the IED of RUP by almost 10 fold (43.7% vs. 4.40%) in the rye grain (quickly and extensively degraded) compared with the corn grain (poorly degraded). As the digestion site (rumen or small intestine) affects the quantity of feed protein absorbed as amino acids, it is important to correct for ruminal microbial colonization in both rumen and intestine to obtain accurate evaluations of protein digestion. In this way, our results were obtained considering both k_c and k_p rates to take into account the real rumen residence time of feed particles. As a consequence of the extended degradation with the increase of the considered rumen residence time, the use of k_c is associated not only with a reduction of the estimates of the by-pass fraction but also with a higher microbial colonization and enrichment in indigestible compounds of this by-pass, which also affect IED estimates^{2,15}. Both microbial noncorrected and corrected values of IED were lower than the predictions of the INRA⁴ and NRC³ systems for protein intestinal digestibility, with the exception of the DDGS of wheat (values for DDGS of barley are not available in the literature) and of palm kernel. Thus, these predictions for corn grain, rye grain, wheat grain, corn gluten feed, DDGS of wheat, sugar beet pulp, rape seed and palm kernel were respectively: 0.88, 0.90, 0.92, 0.85, 0.85, 0.80, 0.85, 0.79 and 0.79 (INRA⁴) and 0.90, 0.80, 0.95, 0.85, 0.80, 0.75, 0.80, 0.75 and 0.75 (NRC³). Nevertheless, these systems omit the microbial colonization correction as well as k_c and, as previously discussed, this results in an overestimation of the intestinal digestibility.

The lack of effect of the ruminal microbial colonization on the regression between IDP and RUP in spite of the large variation of the resulting overestimation of IDP values (from 5.33% in wheat distillers dried grains with solubles to 75.4% in rye grain) can be attributed to the large intestinal digestion of the CP of adherent microorganisms. Thus, the associated increases of CP in RUP estimates due to microbial colonization are largely eliminated by the intestinal digestion. The previously discussed modulated effects of microbial colonization correction, associated with the feed characteristics, on degradability and content of intestinal undigestible components may also contribute to the lack of this effect. The high determination coefficient ($R^2 = 0.960$) found for this prediction shows that values for protein rationing, such as IDP, may be predicted from RUP, simplifying the feed protein value determination which can be limited to the ruminal degradation studies. Microbial corrected and noncorrected data may be used together for this prediction, improving its accuracy.

4. Conclusions

Ruminal and intestinal *in situ* digestion studies should consider ruminal microbial colonization to avoid errors regarding the digestion site when considering accurate protein evaluations of feedstuffs. The close relationship found between the contents of intestinal digested CP and ruminal undegraded CP, which is not affected by this microbial colonization, allows a simpler evaluation of feed protein.

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Table 1: Chemical fractions^a (g kg⁻¹ dry matter unless otherwise stated) in tested feeds and diet ingredients

Feeds	DM (%)	OM	CP	Lipids	NDF	ADF	Lignin	NDIN ^b	ADIN ^b
Corn grain	905	987	87.8	33.8	98.8	19.0	5.41	77.7	9.20
Rye grain	874	985	126	15.5	139	32.8	5.62	93.4	14.4
Wheat grain	878	984	137	20.1	128	33.7	6.53	77.5	7.50
Corn gluten feed	923	931	175	27.1	371	104	8.13	83.3	23.3
DDGS ^c barley	910	949	232	58.5	457	159	47.8	347	55.8
DDGS wheat	891	950	346	75.0	454	121	46.1	426	55.8
Wheat bran	891	945	138	32.7	411	147	45.5	265	57.9
Sugar beet pulp	908	952	99.0	5.60	474	234	39.6	365	68.0
Rapeseed meal	916	917	350	29.2	338	252	121	239	137
Palm kernel	915	953	141	92.8	651	374	104	756	221
Diet-oat hay	904	889	129	-	536	301	48.8	-	-
Diet-concentrate	882	937	149	-	135	43.0	7.81	-	-

^aDM, Dry matter; OM, Organic matter; CP, Crude protein; NDF and ADF, Neutral and acid detergent fibre, respectively; NDIN and ADIN, N insoluble in neutral and acid fibre solutions, respectively.

^bg N kg⁻¹ N

^cDDGS, Distillers' dried grains with solubles.

Table 2. Mean data of the ruminal microbial contamination (RMC), the ruminally undegraded crude protein fraction (RUP), its intestinal effective digestibility (IED) and the intestinal digested crude protein (IDP) noncorrected (NC) and corrected (C) by RMC of different feeds (values estimated based on particle comminution (k_c ; 0.734 h^{-1}) and outflow (k_p ; 0.0450 h^{-1}) rates and expressed as proportions).

	RMC ^d	RUP		IED of RUP		IDP	
		NC	C	NC	C	NC	C
Corn grain ^a	0.187	0.185	0.151	0.830	0.795	0.154	0.120
Rye grain ^a	0.179	0.059	0.048	0.408	0.284	0.024	0.014
Wheat grain ^a	0.161	0.047	0.040	0.550	0.473	0.026	0.019
Corn gluten feed ^a	0.146	0.181	0.155	0.648	0.586	0.117	0.091
DDGS barley ^a	0.065	0.248	0.232	0.849	0.843	0.211	0.196
DDGS wheat ^a	0.036	0.353	0.340	0.911	0.898	0.322	0.305
Wheat bran ^a	0.145	0.222	0.191	0.587	0.516	0.130	0.099
Sugar beet pulp ^b	0.201	0.466	0.372	0.679	0.603	0.316	0.224
Rapeseed meal ^c	0.046	0.408	0.389	0.709	0.691	0.289	0.269
Palm kernel ^c	0.064	0.685	0.641	0.761	0.749	0.521	0.480

^{a, b and c} Data from references 2, 13 and 15, respectively.

^d as proportion of RUP.

Figure 1. Relationship in ten concentrates and by-products between the intestinal digested protein (IDP) and the ruminal undegraded protein (RUP). $IDP = -0.0132 (\pm 0.006795) + 0.776 (\pm 0.0002) RUP$; $n = 60$; $P < 0.001$; $R^2 = 0.960$. Values corrected (○) or noncorrected (●) for the microbial colonization in the rumen. Probability of other effects: colonization correction: $P = 0.682$; colonization correction $\times$ RUP: $P = 0.991$.