UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS

POTENCIACIÓN DE LA RESPUESTA PRODUCTIVA EN RUMIANTES MEDIANTE EL EMPLEO DE PROTEÍNAS PROTEGIDAS

TESIS DOCTORAL

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DEPARTAMENTO DE PRODUCCIÓN AGRARIA

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Dedicatoria

A mi mujer Nuria

RESUMEN

El principal objetivo de esta tesis fue incrementar la eficiencia proteica en las dietas de rumiantes mediante el uso de proteínas protegidas (harina de girasol y guisante de primavera), así como mejorar la predicción de los aportes de proteína microbiana.

Una partida de harinas comerciales de girasol (HG) y de guisante de primavera (GP) fueron tratadas con soluciones 4 N de ácido málico (268,2 g/L) o ácido ortofosfórico (130,6 g/L). Para cada harina, ácido y día de tratamiento, dos fracciones de 12,5 kg fueron pulverizadas sucesivamente en una hormigonera con la solución de ácido correspondiente mediante un pulverizador de campo. Las dos fracciones fueron mezcladas posteriormente y se dejaron reposar durante 1 h a temperatura ambiente. La mezcla fue luego secada en una estufa de aire forzado a 120 ºC durante 1 h. La estufa fue apagada inmediatamente después y el material tratado se mantuvo dentro de ésta hasta la mañana siguiente. El material fue removido durante el proceso de secado cada 30 min durante las primeras 2 h y cada 60 min durante las 5 h posteriores. Este proceso se repitió hasta conseguir las cantidades de harinas tratadas necesarias en los distintos ensayos.

En el primer experimento (capitulo 3) se llevaron a cabo estudios de digestión ruminal e intestinal para evaluar los efectos de la aplicación de las soluciones ácidas indicadas y calor a fin de proteger las proteínas de HG y GP contra la degradación ruminal. Estos estudios se realizaron con tres corderos canulados en el rumen y en el duodeno. El estudio de digestión ruminal fue realizado en tres periodos experimentales en los que los corderos fueron alimentados sucesivamente con tres dietas isoproteicas que incluían HG y GP, sin tratar o tratadas con ácidos málico u ortofosfórico. Cada periodo experimental de 21 días incluyó sucesivamente: 10 días de adaptación a las dietas, un estudio del tránsito ruminal de las partículas de HG y GP (días 11 a 14), y la incubación de las muestras de ambos alimentos en bolsas de nailon (días 15–21). Las harinas incubadas en cada periodo experimental correspondieron a las que fueron incluidas en las dietas. Las bacterias ruminales fueron

marcadas desde el día 11 hasta el día 21 del periodo experimental mediante infusión intra-ruminal continua con una fuente de ^{15}N. Tras finalizar las incubaciones in situ el día 21 el rumen fue vaciado en cada periodo para aislar las bacterias asociadas a la fase sólida y liquida del rumen. El estudio de digestión intestinal fue realizado veinte días después del final del estudio ruminal a fin de eliminar el enriquecimiento en ^{15}N de la digesta. En este estudio se incubaron muestras compuestas obtenidas mediante la combinación de los diferentes residuos no degradados en el rumen de forma que fuesen representativas de la composición química de la fracción no degradada en el rumen (RU). En esta fase los corderos fueron alimentados con la dieta sin tratar para determinar la digestibilidad de las harinas tanto tratadas como sin tratar mediante la técnica de las bolsas móviles. Además, las proteínas contenidas en las harinas tratadas y sin tratar, así como en las muestras correspondientes a los residuos a 0 h, las muestras compuestas anteriormente indicadas y las muestras no digeridas intestinalmente fueron extraídas y sometidas a electroforesis para determinar el sitio de digestión de las diferentes fracciones proteicas.

Las estimaciones de la RU y la digestibilidad intestinal de la materia seca, la materia orgánica (solamente para RU), la proteína bruta (PB) y el almidón (solamente en GP) fueron obtenidos considerando la contaminación microbiana y las tasas de conminución y salida de partículas. Las estimaciones de RU y de la digestibilidad intestinal disminuyeron en todas las fracciones evaluadas de ambos alimentos al corregir por la contaminación microbiana acaecida en el rumen. Todas las estimaciones de RU aumentaron con los tratamientos de protección, incrementándose también la digestibilidad intestinal de la materia seca en la HG. Los bajos valores de la digestibilidad de la proteína de GP tratado y sin tratar sugieren la presencia de algún factor antitripsico no termolábil es esta harina. Los tratamientos de protección incrementaron consistentemente la fracción de materia seca y PB digerida intestinalmente en los dos alimentos, mientras que la fracción de almidón en la muestra de GP solamente aumentó

numéricamente (60,5% de media). Sin embargo, los tratamientos también redujeron la fermentación de la materia orgánica, lo cual podría disminuir la síntesis de proteína microbiana. Los estudios de electroforesis muestran la práctica desaparición de la albumina por la degradación ruminal en ambos alimentos, así como que los cambios en otras proteínas de la muestra RU fueron más pronunciados en GP que en HG.

La composición de las bacterias asociadas con las fases de digesta ruminal sólida (BAS) y líquida (BAL) fue estudiada para revisar la precisión de un sistema de predicción previo que determinaba la infravaloración del aporte de nutrientes correspondiente a las BAS cuando de usa ^{15}N como marcador y las BAL como referencia microbiana (capitulo 4). Al comparar con BAS, BAL mostraron menores contenidos en materia orgánica, polisacáridos de glucosa y lípidos totales y un mayor contenido en PB, así como un mayor enriquecimiento en ^{15}N. Los datos obtenidos en el estudio actual se ajustan bien a la ecuación previa que predice el enriquecimiento en ^{15}N de las BAS a partir del mismo valor en BAL. Esta nueva ecuación permite establecer que se produce una infravaloración de un 22% en el aporte de PB al animal a partir de las BAS sintetizadas si las BAL son usadas como muestras de referencia. Una segunda relación calculada utilizando los valores medios por dieta expuestos en numerosos trabajos encontrados en la literatura confirma la magnitud de este error. Esta infravaloración asociada al uso de BAL como referencia fue mayor para el aporte de glucosa (43,1%) y todavía mayor para el aporte de lípidos (59,9%), como consecuencia de los menores contenidos de ambas fracciones en BAL frente a SAB. Estos errores deberían ser considerados para obtener mayor precisión en la estimación del aporte de nutrientes microbianos y mejorar la nutrición de los rumiantes.

En el experimento 2 se realizó un estudio de producción (capitulo 5) para evaluar los efectos del tratamiento de las harinas HG y GP con soluciones de ácido málico o ácido ortofosfórico sobre el crecimiento, el consumo de concentrado y el rendimiento y

engrasamiento de las canales de corderos de engorde. Noventa corderos machos de cruce entrefino procedentes de tres granjas comerciales (peso inicial medio = 14,6, 15,3 y 13,3 kg, respectivamente) fueron asignados aleatoriamente a cinco dietas con diferentes niveles de proteína y diferentes tratamientos con ácidos y engordados hasta un peso medio al sacrificio de 25 kg. Las fuentes de proteína en el pienso control (C; PB=18,0%) fueron harina de soja, HG y GP sin tratar. En tres de los piensos experimentales, las harinas tratadas con ácido ortofosfórico sustituyeron a las de HG y GP sin tratar (Control Ortofosfórico, PC; PB=18,0% sobre materia seca), sustituyéndose, además, la harina de soja parcialmente (Sustitución Media Ortofosfórico, MSP; PB=16,7%) o totalmente (Sustitución Total Ortofosfórico, TSP; PB=15,6%). Finalmente, en uno de los piensos el ácido ortofosfórico fue reemplazo por acido málico para proteger ambas harinas (Sustitución Media Málico, MSM; PB= 16,7%). La paja de trigo (fuente de forraje) y el concentrado fueron ofrecidos ad libitum. Dieciocho corderos fueron distribuidos en seis cubículos con tres animales para cada dieta. Los datos fueron analizados según un análisis factorial considerando el peso inicial como covariable y la granja de procedencia como bloque. Los datos de consumo de concentrado y eficiencia de conversión fueron analizados usando el cubículo como unidad experimental, mientras que los datos sobre ganancia media diaria, rendimiento a la canal, grasa dorsal y grasa pélvico renal fueron analizados usando el cordero como unidad experimental. No se encontró ningún efecto asociado con el nivel de PB sobre ninguna variable estudiada. Esto sugiere que usando proteínas protegidas es posible utilizar concentrados con 15,6% de PB (sobre materia seca) disminuyendo así la cantidad de concentrados de proteína vegetal a incluir en los piensos y la calidad de los concentrados proteicos. Los corderos alimentados con la dieta MSM tuvieron mayores ganancias medias diarias (15,2%; P= 0,042), y mejores rendimiento a la canal en caliente (1,3 unidades porcentuales; P= 0,037) que los corderos alimentados con el concentrado

MSP. Esto podría ser explicado por los efectos benéficos ruminales del malato o por el mayor efecto de protección conseguido con el ácido málico.

SUMMARY

The main objective of this thesis project was to increase the protein efficiency in ruminant diets by using protected protein (sunflower meal and spring pea), and improving the prediction of microbial protein supply.

Commercial sunflower meal (SFM) and spring pea (SP) were treated with 4 N solutions (200 mL/kg) of malic acid (268.2 g/L) or orthophosphoric acid (130.6 g/L). Daily, two fractions of 12.5 kg of one of these meals were successively sprayed with the tested acid solution in a concrete mixer using a sprayer. Both fractions were then mixed and allowed to rest for 1 h at room temperature. The blend was then dried in a forced air oven at 120 ºC for 1 h. Then the oven was turned off and the treated material was left in the oven overnight. During the drying process, the material was stirred every 30 min during the first 2 h and then every 60 min for the subsequent 5 h. This process was repeated until the amounts of treated flour needed for the different trials performed.

In the first experiment (chapter 3), ruminal and intestinal digestion trials were conducted to study the effects of the application of these acid solutions and heat to protect proteins of SFM and SP against ruminal degradation using three wethers fitted with rumen and duodenum cannulae. The ruminal digestion study was carried out in three experimental periods in which the wethers were successively fed three isoproteic diets including SFM and SP, untreated or treated with malic or orthophosphoric acids. The experimental periods of 21 days included successively: 10 days of diet adaptation, SFM and SP particle ruminal transit study (days 11–14) and ruminal nylon-bag incubations (days 15–21). The meals incubated in each experimental period were those corresponding to the associated diet. Rumen bacteria were labelled from days 11 to 21 by continuous intra-ruminal infusion of a ^{15}N source and the rumen was emptied at the end of in situ incubations in each period to isolate solid adherent bacteria and liquid associate bacteria. The intestinal digestion trial was conducted twenty days after the end of the ruminal studies to eliminate the ^{15}N enrichment in the digesta. The tested samples

were composite samples obtained pooling the different ruminally undegraded residues to be representative of the chemical composition of the ruminally undegraded fraction (RU). Wethers were fed the untreated diet to determine the intestinal digestibility of untreated and treated meals using the mobile nylon bag technique. In addition, protein in untreated and treated meals and their 0 h, composite and intestinally undigested samples were extracted and subjected to electrophoresis to determine the digestion site of the different protein fractions.

Estimates of the RU and its intestinal digestibility of dry matter, organic matter (only for RU), crude protein (CP) and starch (only in SP) were obtained considering ruminal microbial contamination and particle comminution and outflow rates. When corrected for the microbial contamination taking place in the rumen, estimates of RU and intestinal digestibility decreased in all tested fractions for both feeds. All RU estimates increased with the protective treatments, whereas intestinal digestibility-dry matter also increased in SFM. Low intestinal digestibility-CP values in untreated and treated samples suggested the presence of non-heat labile antitrypsin factors in SP. Protective treatments of both feeds led to consistent increases in the intestinal digested fraction of dry matter and CP, being only numerically different for SP-starch (60.5% as average). However, treatments also reduced the organic matter fermentation, which may decrease ruminal microbial protein synthesis. Electrophoretic studies showed albumin disappearance in both SFM and SP, whereas changes in other RU proteins were more pronounced in SP than SFM.

The chemical composition of bacteria associated with solid (SAB) and liquid (LAB) rumen-digesta phases was studied to examine the accuracy of a previous regression system determining the underevaluation of SAB-nutrient supply using ^{15}N as marker and LAB as microbial reference (chapter 4). Compared with SAB, LAB showed lower contents of organic matter, polysaccharide-glucose and total lipids and the opposite for the CP content and the ^{15}N enrichment. Present data fitted well to the previous relationship predicting the ^{15}N enrichment

of SAB from the same value in LAB. This new equation allows establishing an underevaluation in the supply of CP from the synthesized SAB in 22.0% if LAB is used as reference. Another relationship calculated using mean diet values from the literature confirmed the magnitude of this error. This underevaluation was higher for the supply of glucose (43.1%) and still higher for the lipid supply (59.9%) as a consequence of the lower contents of these both fractions in LAB than in SAB. These errors should be considered to obtain more accurate estimates of the microbial nutrient supply and to improve ruminant nutrition.

A production study was performed in experiment 2 (chapter 5) to examine the effects of treating SFM and SP meals with orthophosphoric or malic acid solutions on growth performance, concentrate intake, and carcass yield and fatness of growing-fattening lambs. Ninety "Entrefino" cross male lambs from three commercial farms (average initial body weights (BW) = 14.6, 15.3 and 13.3 kg) were randomly assigned to five diets with different acid treatment and protein levels, and fattened to an average slaughter weight of 25 kg. Protein sources in the control concentrate (C; CP=18%) were soybean meal and untreated SFM and SP. In three of the experimental concentrates, orthophosphoric acid-treated meals substituted untreated SFM and SP (Orthophosphoric Control, PC; CP=18% dry matter basis), and soybean meal was partially (Medium Substitution Orthophosphoric, MSP; CP=16.7%) or totally removed (Total Substitution Orthophosphoric, TSP; CP=15.6%). In addition, in one concentrate orthophosphoric acid was replaced by malic acid to protect these meals (Medium Substitution Malic, MSM; CP= 16.7%). Wheat straw (roughage source) and concentrate were offered ad libitum. Eighteen lambs were allocated to six pens of three animals on each diet. Data were analyzed using a factorial analysis with initial body weight BW as covariate and farm of origin as block. Data on concentrate intake and feed conversion efficiency were analyzed using pen as experimental unit, while data on average daily gain, carcass yield, dorsal fat, and kidney-pelvic-fat were analyzed with lamb as experimental unit. No effect associated

with the CP level was observed on any parameter. This suggests that with protected proteins it is possible to feed concentrates with 15.6% CP (dry matter basis) reducing the quantity of vegetable protein meals to include in the concentrate as well as the quality of the protein concentrates. Lambs feed MSM had higher average daily gains (15.2%; *P*= 0.042), and better hot carcass yields (1.3 percentage points; *P*= 0.037) than lambs feed MSP. This probably can be explained by ruminal malate actions and by greater protection effects obtained with malic acid.

ABREVIATURAS

AA: Aminoácidos

ADIN: Nitrógeno insoluble en solución de fibra ácido detergente

AGV: Ácidos grasos volátiles

BAL: Bacterias asociadas a la fracción líquida

BAS: Bacterias asociadas a la fracción sólida

BOE: Boletín Oficial del Estado

C: Pienso control

d: Día

EUN: Eficiencia de utilización del nitrógeno

FEDNA: Fundación española para el desarrollo de la nutrición animal

GP: Guisante de primavera

GMD: Ganancia media diaria

h: Hora

ha: hectárea

HG: Harina de girasol

IC: Índice de conversión

M: Molaridad

min: Minuto

MO: Materia orgánica

MSM: Pienso sustitución media málico

MSP: Pienso sustitución media ortofosfórico

N: Nitrogeno

NDIN: Nitrógeno insoluble en solución de fibra neutro detergente

NNP: Nitrógeno no proteico

P: Probabilidad

PB: Proteína bruta

PC: Pienso control ortofosfórico

PDR: Proteína degradable en rumen

PM: Proteína bruta microbiana

PNDR: Proteína no degradable en el rumen

RU: Fracción no degradada en el rumen

t: Tonelada

TSP: Pienso sustitución total ortofosfórico

ABBREVIATIONS

ADF: Acid detergent fiber

ADG: Average daily gain

ADIN: Insoluble nitrogen in acid detergent fibre solution

AOAC: Association of Official Analytical Chemists

BW: Body weight

C: Control concentrate

CCY: Cold carcass yield

CP: Crude protein

CS: Composite samples

d: Day

DF: Dorsal fat

DM: Dry matter

Eq: Equivalent weight

FCR: Feed conversion ratio

h: Hour

HCY: Hot carcass yield

IADF: Intestinal absorbable dietary fraction

ID: Intestinal digestibility

k_c: Fraccional rate of particle comminution and mixing in the rumen

k_d: Fraccional degradation rate of nonsoluble degradable fraction

KD: Kilodalton

k_p: Fraccional rate of particle outflow from the rumen

KPF: Kidney pelvic fat

LAB: Liquid associated bacteria

M: Molarity

Min: Minute

MSM: Medium substitution malic concentrate concentrate

MSP: Medium substitution orthophosphoric concentrate

MT: Diet including protein concentrates treated with malic acid

MW: Molecular weights

NDF: Neutral detergent fiber

NDIN: Insoluble nitrogen in neutral detergent fiber solution

NRC: National Research Council

OM: Organic matter

P: Probability

CP: Control orthophosphoric concentrate

PT: Diet including protein concentrates treated with orthophosphoric acid

RSD: Residual Standard Deviation

RU: Ruminally undegraded fraccion

s.e.m.: Standard error of the mean

SAB: Solid adherent bacteria

SFM: Sunflower meal

SP: Spring pea

t: Ton

TSP: Total substitution orthophosphoric concentrate

UFV: “Unites Fourrageres Viande”

UT: Diet including untreated protein concentrates

v: Volume

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CAPITULO 1

ALIMENTACIÓN NITROGENADA DEL RUMIANTE. EFICIENCIA Y MEDIO AMBIENTE: REVISIÓN BIBLIOGRÁFICA

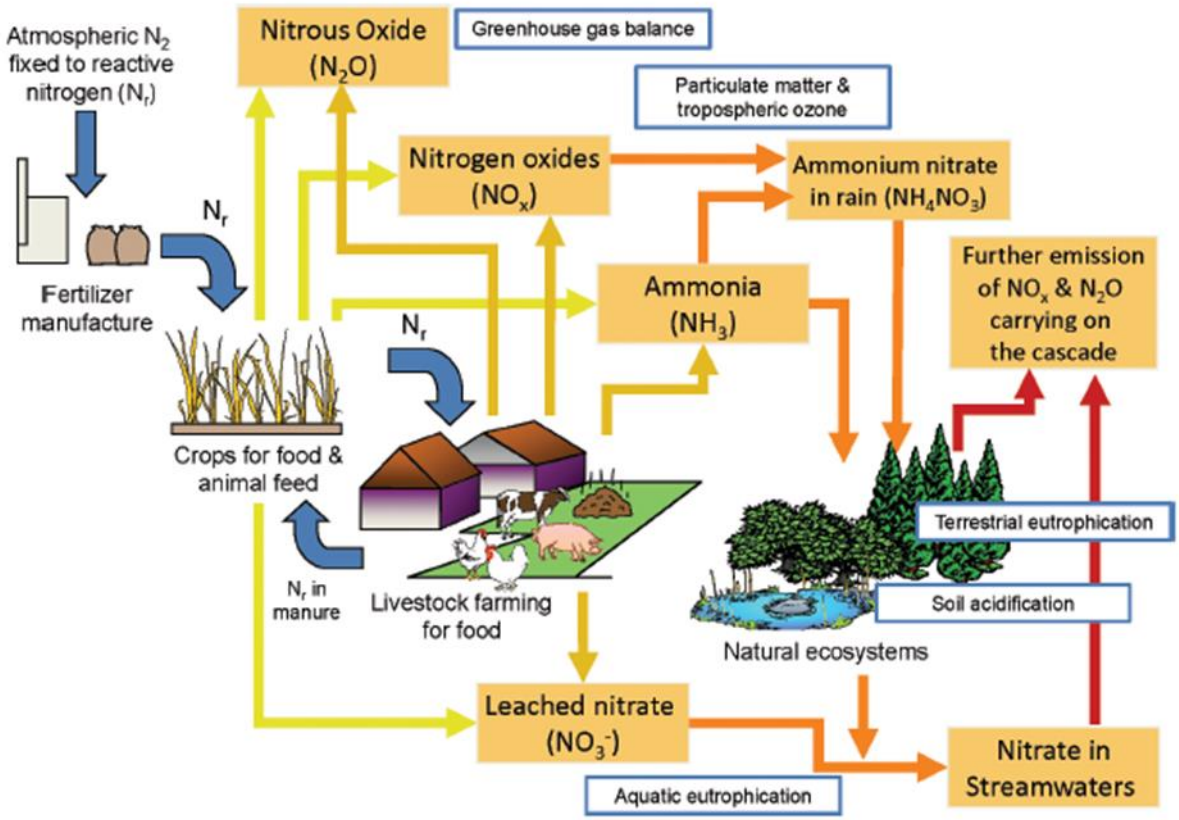
1.1.Contaminación ambiental por nitrógeno en los sistemas de producción animal

Uno de las principales problemas medioambientales es la emisión de nitrógeno (N) al ambiente (Dijkstra y col., 2013). El N es un elemento químico que no puede ser ni producido ni destruido por el metabolismo animal y solo pueden ser transformadas las moléculas que lo contienen (Pfeffer y Hristov, 2005). La mayoría del N consumido por los animales es excretado, actuando como nutriente necesario para el crecimiento de las plantas; sin embargo el principal problema durante este ciclo del N es que se producen perdidas elevadas de N que contribuyen a la degradación del medioambiente (Rotz, 2004). Las mayores pérdidas de N que ocurren en los sistemas intensivos de producción animal se producen mediante las emisiones de gases a la atmosfera y la escorrentía de nitratos (NO_3^-) a aguas superficiales y subterráneas (figura 1). A continuación se detallan los principales contaminantes atmosféricos originados en los sistemas de producción animal (NRC, 2003):

- Amoniaco (NH_3): La urea presente en la orina de los animales es hidrolizada a NH_3 y dióxido de carbono lo que se facilita por las enzimas ureasas que se encuentran en las heces. Una vez que ha sido emitido, el NH_3 puede ser convertido rápidamente a ion amonio ($NH_4^{+)}$. El NH_4^+ contribuye a la eutrofización, acidificación, y fertilización de los ecosistemas. Un 48% de las emisiones de NH_3 al ambiente son producidas por los animales de producción.
- Óxido nitroso (N_2O): El N_2O es formado y emitido a la atmosfera mediante los procesos microbianos de nitrificación y des-nitrificación ocurridos en el suelo. Este gas produce el calentamiento de la troposfera y pérdidas de ozono en la estratosfera. Un tercio de este contaminante procede de granjas animales.
- Óxido nítrico (NO): NO y dióxido de nitrógeno (NO_2) son inter convertidos rápidamente en la atmosfera y por eso son referidos en conjunto como NO_X. Las

emisiones de este gas procedentes de los animales y sus excretas son muy bajas, representando un 1% de las emisiones totales.

Figura 1: Resumen de las pérdidas de N en un modelo de producción intensivo:



Fuente: Sutton, 2011.

Los rumiantes juegan un papel principal en el suministro de alimentos mediante la conversión de productos fibrosos incomestibles para los humanos en alimentos de alta calidad; sin embargo esta conversión produce inevitables pérdidas de N en las heces y orina que pueden producir un alto impacto medioambiental (Dijkstra y col., 2013). Así, la contribución de los rumiantes a las emisiones globales de amoniaco es superior al resto de especies de producción (Bouwman y col., 1997).

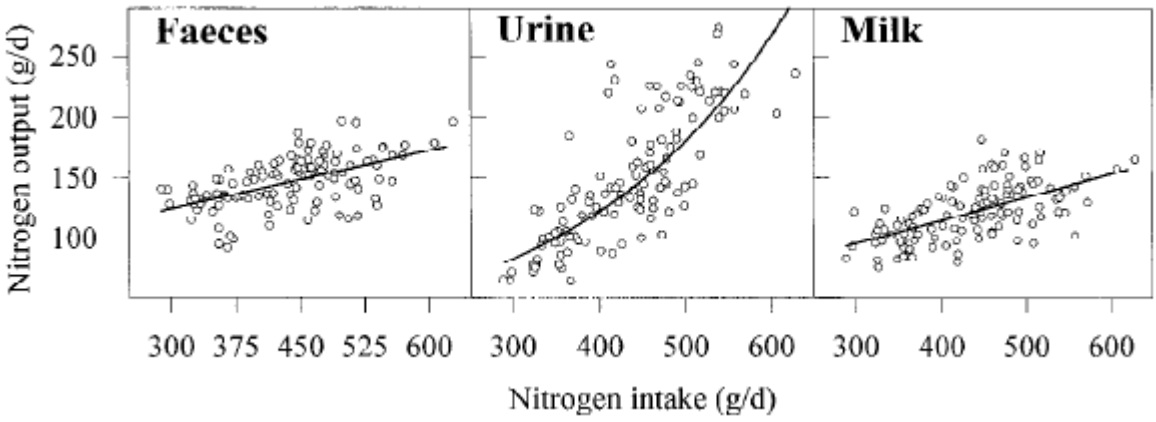
Las pérdidas de N pueden ser reducidas mediante la mejora de la eficiencia del animal para usar la proteína alimentaria, disminuyendo las pérdidas durante el almacenamiento y el manejo de las excretas, y haciendo rotación de cultivos para aprovechar mejor el N de las excretas; de esas 3 opciones, la primera es la más rápida y fácil de implementar (Rotz y col., 1999).

No hay ninguna duda de que en el ganado de leche y de carne el consumo total de N es el factor más importante que afecta la excreción de N en las excretas (heces más orina; Reynolds y col., 2010). En ganado vacuno lechero, el consumo de N fue identificado como el principal factor responsable de la excreción de N. Así, disminuyendo su contenido en las dietas se puede reducir significativamente la excreción total de N (Kebreab y col., 2010; Satter y col., 2002). La dieta no solo puede afectar la cantidad de N excretado por los rumiantes, sino también la proporción relativa de éste que es excretado en orina o heces. En dietas bajas en proteína, el N fecal representa una mayor proporción del N consumido (valores máximos del 50%) que el N de la orina (valores mínimos del 25%), sin embargo, en cuanto la proteína dietaría aumenta, la contribución del N fecal disminuye y la excreción del N urinario puede aumentar rápidamente y llegar a representar un 60% del N total consumido (Dijkstra y col., 2011).

Usando datos de 5 estudios llevados a cabo con vacas lecheras, investigadores de la Universidad de Reading (UK, Kebreab y col., 2001) desarrollaron un modelo matemático para predecir la cantidad y la forma del N excretado en diferentes sistemas de manejo productivo. Estos autores encontraron que mientras el N excretado en leche o heces estaba relacionado positivamente y de forma lineal con el N consumido, el N excretado en orina estaba relacionado exponencialmente con el consumo de N, aumentando el ritmo de excreción de N drásticamente cuando el consumo de N ronda los 400 g por día (figura 2). Olmos Colmenero y Broderick (2006) también indicaron que la proporción de N consumido excretado en orina incrementó

linealmente de 23,8 a 36,2% cuando el contenido de N en dietas de vacas lecheras aumentó de 13,5 a 19,4%.

Figura 2: Relación entre el consumo de N por vacas lecheras y la excreción de éste en heces, orina y leche



Fuente: Kebreab y col., 2001.

El N excretado en las heces es más estable y tiene menos probabilidad de ser volatizado a la atmosfera que el N excretado en orina, ya que gran parte de este último se encuentra en forma de urea, y la urea puede ser rápidamente hidrolizada a NH_3 por las enzimas ureasas (Satter y col., 2002). Por lo tanto, toda estrategia de alimentación de rumiantes debería enfocarse en disminuir las pérdidas de N urinario como objetivo prioritario.

Varios estudios (Kebreab y col., 2001; Olmos Colmenero y Broderick, 2006) demuestran la importancia de no suministrar proteína por encima de las necesidades del animal. Sin embargo, las formulaciones prácticas de proteína suelen ser superiores a las recomendaciones de los distintos sistemas de racionamiento. Resultados de una encuesta realizada en 372 granjas lecheras localizadas en la Bahía de Chesapeake (EEUU) demostraron que los productores estaban suministrando un 6,6% más de N que el recomendado por el National Research Council (NRC, 1989), y como resultado, la excreción de N en orina y heces

estaba siendo aumentada en un 16,0 y 2,7%, respectivamente (Jonker y col., 2002). Similares resultados fueron encontrados en ganado vacuno de engorde. Galyean (1996) encontró que los niveles de proteína bruta que estaban siendo utilizados en terneros de engorde en fase de acabado eran mayores a los recomendados por el NRC. Entre los motivos que justifican esta sobrealimentación proteica en estos animales se citan: el estrés de los animales recién llegados al cebadero, que disminuye la ingestión y aumenta el catabolismo proteico, la variabilidad dentro de los lotes de cebo, con animales de diferente edad, sexo, genética, etc., y por lo tanto diferentes potenciales de crecimiento y capacidades de ingestión, y el uso de factores de seguridad frente a las diferencias de calidad de las materias primas que intervienen en la elaboración del pienso (INRA,1988; Ferret y col., 2006).

1.2. Eficiencia en la utilización del nitrógeno en los rumiantes

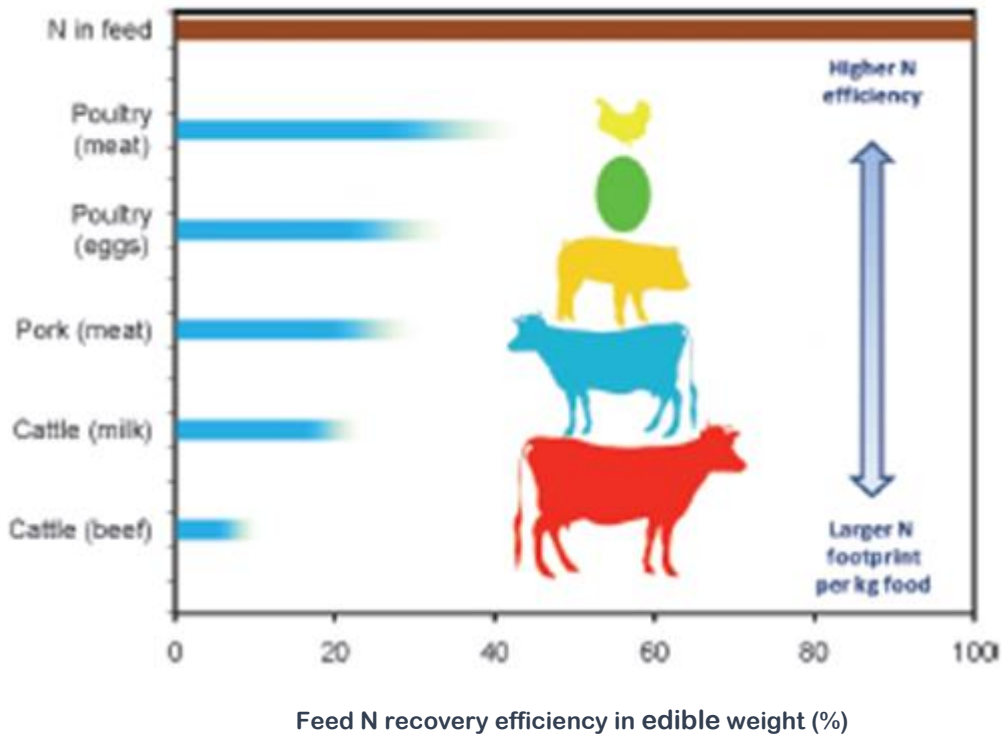
Pese a que el rumen representa ventajas, sobre todo cuando los rumiantes son alimentados con productos de baja calidad, la digestión en este compartimento puede ser una de las mayores causas de la baja eficiencia de utilización del N (EUN) en rumiantes (Dewhurst y col., 2000), la cual típicamente promedia un 25% y además es muy variable (10 – 40%; Calsamiglia y col., 2010). En otros animales de producción, como los cerdos y las aves, en los que las necesidades proteicas pueden cubrirse más fácilmente, la EUN promedia entre 30 a 35%, e incluso puede llegar al 40% (Rotz, 2004).

La figura 3 representa gráficamente la EUN de diferentes especies productivas en Europa (Sutton y van Grinsven, 2011). En esta se puede observar que los rumiantes de aptitud cárnica son los animales menos eficientes, mientras que las aves de carne presentan la mayor eficiencia.

En esta misma línea, Jensen y Schjoerring (2011) asignaron las siguientes eficiencias proteicas a diferentes especies de rumiantes en sistema de alojamiento intensivo:

- Ganado vacuno lechero: vacas en lactación 23,3%, novillas de reemplazo de más y menos de 1 año 6,4% y 14,0% respectivamente.
- Ganado vacuno de carne: vacas amamantando 11,4%, terneros de engorde de más y menos de 1 año15,7% y 30,7%, respectivamente.
- Ovejas (incluyendo corderos): 13,5%
- Cabras (incluyendo cabritos): 25,2%

Figura 3: Eficiencia de utilización de N en diferentes especies productivas de Europa



Fuente: Sutton y van Grinsven, 2011.

Usando datos de estudios publicados previamente en revistas científicas, Calsamiglia y col. (2010) encontraron que las medias correspondientes al mayor y al menor cuartil en EUN correspondió a 32,8 y 22,0% en 167 dietas de vacas lecheras de EEUU y 32,0 y 21,0% en 287 dietas europeas, respectivamente. Un meta-análisis publicado por Huhtanen y Hristov (2009) demostró que la eficiencia de utilización del N para la síntesis de proteína láctea en vacas lecheras del norte de Europa y Norteamérica fue 27,7 y 24,7%, respectivamente. Además, este

meta-análisis indicó que la concentración de proteína bruta en las dietas es el factor más importante que afecta a la EUN en vacas lecheras y que esta eficacia disminuye al incrementar el consumo de proteína. La EUN también disminuyó linealmente de 36,5 a 25,4% al aumentar el contenido proteico de dietas de vacas lecheras de 13,5 a 19,4% (Olmos Colmenero y Broderick, 2006).

Dijkstra y col. (2013) estimaron que la máxima EUN teórica en una vaca lechera produciendo 40 kg diarios de leche corregida por grasa y proteína es 43%. Las pérdidas teóricas inevitables de N en heces y orina fueron estimadas en 89 y 174 g/día, respectivamente, y las principales causas por las que se producen son: excreción de urea sintetizada a partir del NH_3 procedente del rumen, proteína bruta microbiana (PM) no digerida, excreción de derivados de los ácidos nucleicos sintetizados en el rumen, secreciones endógenas, mantenimiento y síntesis de proteína láctea. Los autores concluyen que hay muy pocas, o ninguna posibilidad de mejorar la EUN mediante la disminución de las pérdidas de N relacionadas con el reciclado de N en el rumen, la digestión intestinal de la PM y los requerimientos de mantenimiento del animal, y que las principales estrategias para reducir las pérdidas de N deberían enfocarse en un aporte adecuado de proteína degradable en rumen (PDR), y una eficiencia optima en la síntesis de proteína láctea a partir de los aminoácidos (AA) absorbidos.

Schwab y col. (2005) propusieron varias estrategias para aumentar la eficiencia de conversión de N alimentario en proteína láctea o cárnica, y reducir las pérdidas de N en los rumiantes:

1. Alimentar con el objetivo de maximizar la síntesis de PM, lo cual incrementa la probabilidad de capturar N reciclado y los productos finales de la degradación de las proteínas en el rumen.

2. Ajustar y balancear el aporte de PDR y proteína no degradable en el rumen (PNDR) para satisfacer los requisitos de ambas fracciones, pero sin excederlas. En este caso ninguna de las fracciones de proteína es sobrealimentada, y el consumo de N es minimizado.
3. Por último, la tercera estrategia es ajustar y balancear el aporte de AA esenciales en las dietas.

1.3. Degradación de la proteína en el rumen

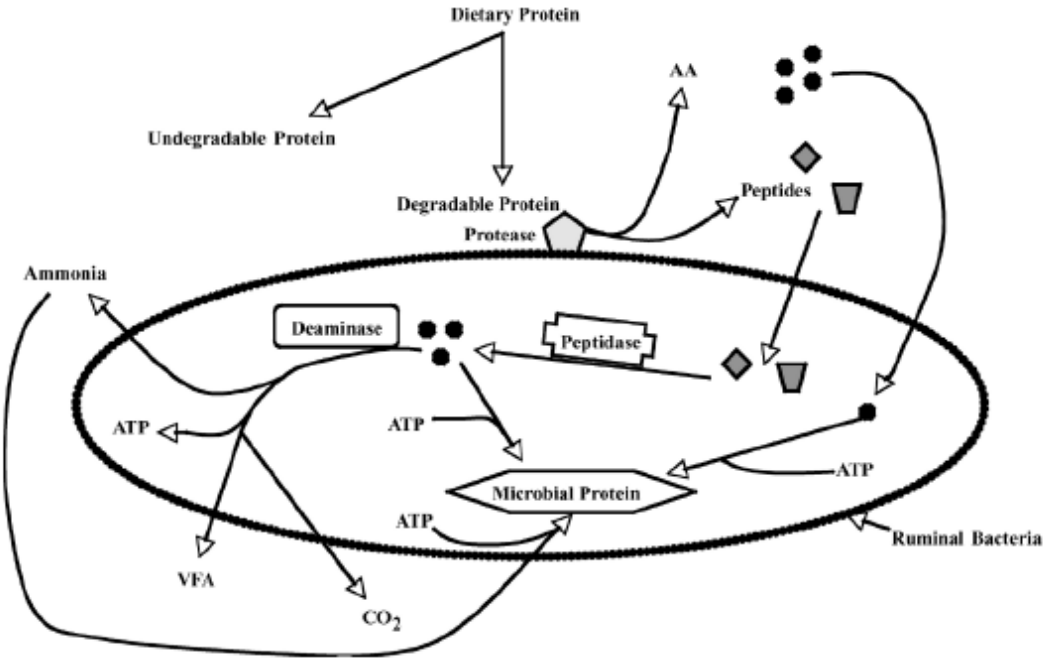
La degradación de la proteína dietética en el rumen es un proceso complejo que involucra muchos microorganismos, los cuales proporcionan las enzimas necesarias para hidrolizar los enlaces peptídicos generando péptidos y AA (Walker y col., 2005). Estos productos de la degradación ruminal son transportados dentro de las células microbianas y siguen las siguientes rutas metabólicas (Bach y col., 2005; Fig. 4):

- Los péptidos son degradados a AA por peptidasas.
- Según la energía (en forma de carbohidratos) disponible en la célula microbiana, los AA pueden ser usados en la síntesis de PM o pueden ser desaminados produciendo NH_3 y esqueletos carbonados, que son fermentados a ácidos grasos volátiles (AGV) y CO_2.

La degradación de las proteínas en los compartimentos estomacales de los rumiantes depende de varios factores, algunos de los cuales están relacionados con las dietas, mientras que otros están relacionados con el animal (Tamminga, 1979). Los principales factores que afectan la cantidad de PB que es degradada en el rumen son: el contenido proporcional de proteínas y nitrógeno no proteico (NNP), las propiedades físicas y químicas de las proteínas, el tiempo de retención de la proteína en el rumen, la actividad proteolítica microbiana, y el pH ruminal (Schwab y col. 2003). Entre todos estos factores, las diferencias en la estructura

tridimensional de las proteínas es el factor más importante que condiciona la extensión y el grado de degradación de las proteínas debido a que afecta el acceso microbiano a estas (NRC, 2001).

Figura 4: Representación esquemática de la degradación de las proteínas en el rumen



Fuente: Bach y col., 2005.

La degradación ruminal de la proteína normalmente provoca una pérdida de proteína neta debido a que la PM está compuesta por un 15 – 20% de ácidos nucleicos, los cuales no están disponibles para el metabolismo del ganado (Tamminga, 1996). Además, la mureina constitutiva de las paredes celulares de las bacterias contiene una elevada proporción de amino-azucares (N-acetil glucosamina y ácido N-acetil murámico) que tampoco pueden ser usados en el metabolismo proteico del animal.

1.3.1. Efecto de la proteína degradable en rumen sobre la excreción de nitrógeno

Cuando las cantidades suministradas de PDR exceden las necesidades de los microorganismos ruminales, se producen cantidades elevadas de NH_3 que son absorbidas en la

sangre, transformadas en urea en el hígado y excretadas en la orina (Olmos Colmenero y Broderick, 2006).

En concordancia con lo ya indicado para el consumo de proteína bruta (PB), la proporción de N excretado en la orina de vacas lecheras incrementó linealmente al aumentar la degradabilidad de la proteína de una dieta compuesta de ensilado de hierba y cantidades diferentes de proteína de soja sin tratar o tratada con formaldehido (Castillo y col., 2001). Además, el ritmo de aumento del N urinario al incrementar la proporción de PDR fue mucho mayor en dietas con alto contenido en proteína que en dietas bajas en proteína.

Varios experimentos realizados en vacuno de leche han demostrado la posibilidad de reducir las emisiones de NH_3 mediante la disminución del aporte de PDR. Agle y col. (2010) evaluaron el efecto de varios niveles de PDR sobre las emisiones de NH_3 en vacas lecheras suministrando 3 dietas que cumplían los requerimientos en proteína metabolizable; sin embargo, una de ellas excedía los requerimientos de PDR en un 7% siendo las otras dos deficientes en este parámetro en un 14 y 27% para su nivel productivo. El N excretado en orina, expresado como porcentaje del N consumido, fue máximo (29,3%) en la dieta con mayor contenido en PDR, mínimo (23,8%) en la dieta con menor contenido de ésta, e intermedio en la dieta con un contenido medio de PDR (26,0%). La proporción del N consumido excretado en heces no fue sin embargo afectada. Además, los autores evaluaron en laboratorio el potencial de emisión de N de las excretas durante 15 días, encontrando que la emisión de NH_3 acumulada durante este periodo fue un 37% inferior en la dieta de menor contenido en PDR que en la de mayor concentración de esta. En concordancia con este experimento, van Duinkerken y col. (2005) reportaron que las emisiones de NH_3 de vacas lecheras alojadas en un sistema de cubículos con ventilación natural se redujeron a la mitad cuando el exceso de RDP en la dieta disminuyó de 1.000 a 0 g/vaca/día.

Estudios recientes de nuestro grupo de investigación (Vanegas y col. 2015 a y b) han demostrado que la degradación ruminal de las proteínas de la dieta se asocia también con generación de metano, probablemente como consecuencia de la fermentación de las cadenas carbonadas resultantes de la desaminación de aminoácidos. Ésta es pues una razón adicional de importancia para tratar de ajustar los aportes de PDR a las necesidades de los microorganismos determinadas por la cuantía y la composición de la ración.

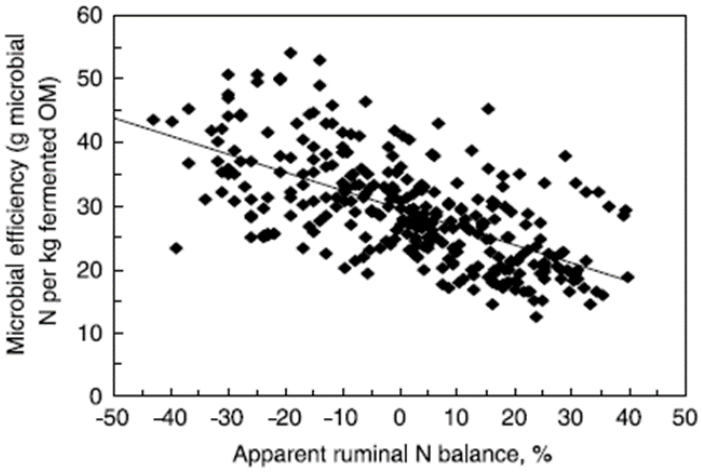
1.4. Síntesis de proteína microbiana

Según el NRC (2001), la producción de PM se estima en 130 g/kg de nutrientes digestibles totales, siempre y cuando el suministro de PDR exceda de 1,18 x PM kg/d, cuando el consumo de PDR es inferior, la producción de PM se estima en 0,85 x PDR consumida. La eficiencia de síntesis de PM (g de N microbiano/kg materia orgánica (MO) fermentable) ha sido estimada en 29,74 cuando el balance ruminal de N es 0, es decir, la disponibilidad de N en el rumen (proporcionada por la dieta y el reciclado) no se encuentra ni en exceso ni en defecto en relación a las necesidades de los microorganismos ruminales (Fig. 5; NRC, 2001). La eficiencia de síntesis de PM esta negativamente correlacionada con la disponibilidad de N ruminal (Fig. 5). Por ejemplo, cuando el balance de N ruminal disminuye de +20 a -20%, la eficiencia de síntesis aumenta de 24 a 36 g N/kg de MO fermentable.

La reducción de la eficacia microbiana al aumentar la disponibilidad de N ruminal indicada por el NRC (2001) se asocia con el menor aprovechamiento energético de la fermentación de la proteína frente a la fermentación de los carbohidratos (Demeyer y Tamminga, 1987; Hvelplund, 1991). De ello se induce el interés de evitar excesos de PDR en las dietas, para potenciar la síntesis microbiana y reducir las fugas de NH_3 del rumen y, por tanto, de urea en la orina.

En el ganado de carne se ha indicado una menor eficiencia en la síntesis de PM que en el ganado lechero, debido probablemente a la elevada población de bacterias amilolíticas, promovida por sus dietas (Stern y col., 2006). Estos autores indican así mismo una menor contribución de la PM a los requerimientos de proteína total, no solo por la razón anterior, sino también por una menor síntesis de PM, debido a la falta de incremento del consumo de materia seca en ganado de carne según incrementa la ganancia media diaria. Además, las dietas tan concentradas utilizadas en el ganado de engorde producen un bajo transito ruminal lo que reduce la eficacia de crecimiento microbiano pues aumenta la lisis microbiana.

Figura 5: Relación entre la eficiencia de síntesis de proteína microbiana y el balance de N ruminal.



Fuente: NRC, 2001. Y= 29,74 - 0,30 X (r^2 = 0,41).

La cuantificación del crecimiento microbiano se puede llevar a cabo únicamente mediante la utilización de marcadores microbianos (Schonhusen y col., 1995), siendo la relación marcador microbiano:N un parámetro fundamental en la estima de la síntesis PM y/o de la eficacia de esta síntesis. La obtención de una muestra representativa de los microorganismos constituye, así, un aspecto fundamental para lograr una adecuada estimación. A este respecto, existen dificultades derivadas de las técnicas de muestreo y aislamiento, así

como del tipo de población microbiana utilizada y su composición. La muestra de microorganismos utilizada como referencia para determinar la síntesis de PM en la mayoría de los estudios in vivo ha correspondido a bacterias aisladas de la fase líquida del rumen, al ser éste el procedimiento más sencillo. Sin embargo, desde hace más de 30 años se sabe que existen importantes diferencias de composición química entre las poblaciones de bacterias asociadas con las fracciones líquida (BAL) y sólida (BAS) del contenido ruminal, especialmente para la relación marcador microbiano: N, cuyos valores son más elevados en BAL que en BAS o en protozoos, sea cual sea el marcador microbiano utilizado (Olubobokun y Craig, 1990; Martin y col.,1994; Pérez y col.,1998; Rodríguez y col.,2000; Yang y col.,2001). Dado que las BAL son marcadamente minoritarias en la digesta ruminal (Merry y McAllan, 1983; Rodríguez el al., 2003), si se utilizan como referencia (como es mayormente el caso de los sistemas actuales de racionamiento basados en datos históricos) se subestima la síntesis de PM y, dado su cálculo por diferencia, se sobrevalora el contenido en proteína by-pass de los alimentos. Estos problemas deben solucionarse, bien mediante el aislamiento de una fracción microbiana representativa de la biomasa que abandona el rumen (lo que es difícil de conseguir) o bien corrigiendo los errores indicados.

1.5. Aportes de PDR y PNDR. Proteínas protegidas

Los concentrados de proteína vegetal más comúnmente utilizados en España en las dietas de rumiantes presentan contenidos en PDR que varían entre el 30 y el 90%, aunque la mayoría de estos la proporción de PDR es superior al 65% de la PB (Fig. 6; FEDNA, 2010). Como se puede apreciar en esta figura, el guisante de primavera es el alimento con mayor porcentaje de PDR (90%), mientras que el gluten "meal" presenta el menor valor de esta fracción proteica (30%). La misma fuente establece también un contenido elevado en PDR para los granos de cereales con valores comprendidos entre 75% y 80%, salvo en maíz (45%) y

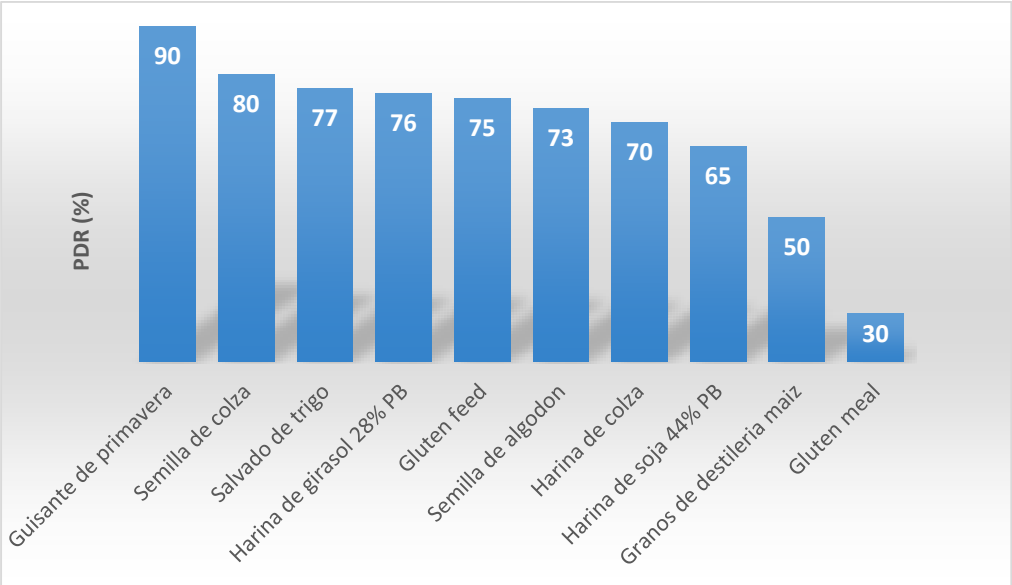
sorgo (43%). Así mismo, las estimaciones in situ de este contenido para la mayor parte de los forrajes son elevadas.

La PDR proporciona una mezcla de péptidos, AA libres, y NH_3 para el crecimiento microbiano y la síntesis de PM (NRC, 2001). La PM representa la mayor parte de la proteína que sale del rumen (55 a 87% del total de N aminoacídico, según Clark y col, 1992), siendo además de muy buena calidad. Sin embargo, generalmente ésta puede ser insuficiente para aportar el total de AA requeridos por los animales de elevada producción. Así, según el NRC (2001) a medida que aumenta la producción, la contribución parcial de la PM al total de AA aportados al intestino disminuye y la cantidad de proteína alimentaria que llega sin degradar al intestino debe aumentar para cubrir las necesidades. En ganado de engorde, la PM puede cubrir el 95% de las necesidades proteicas de un ternero de 250 kg de peso y un crecimiento diario de 300 g, sin embargo solo puede cubrir el 40% de las necesidades cuando la ganancia media de este ternero se incrementa a 1700 g/día (Stern y col., 2006).

Estas estimaciones pueden ser corregidas, sin embargo, teniendo en cuenta las observaciones anteriormente expuestas sobre la subestimación de las estimas de PM en los sistemas actuales de racionamiento basados mayoritariamente en el uso de BAL como referencia y la sobrevaloración asociada del contenido en proteína by-pass de los alimentos, dado su cálculo por diferencia. Ello implicaría una mayor contribución de la PM a la cobertura de las necesidades nitrogenadas, así como una menor concentración en PNDR en los alimentos, lo que revaloriza esta fracción, y un mayor contenido en PDR, que amplifica sus problemas asociados, ya expuestos.

Las proteínas protegidas corresponden a alimentos proteicos que han sido tratados o procesados con la finalidad de disminuir la degradabilidad ruminal de la proteína y aumentar su contenido en PNDR digestible en el intestino (NRC, 2001). Muchos métodos han sido

Figura 6: Valores de proteína degradable en rumen de las principales fuentes de proteína vegetal usadas en España



Fuente: FEDNA, 2010.

investigados para disminuir la fermentación ruminal de concentrados proteicos; la mayoría de estos métodos se basan en la aplicación de calor, agentes químicos o una combinación de ambos que alteran las características de la proteína e incrementan su resistencia a las enzimas proteolíticas (Bróderick y col., 1991).

El calor provoca la desnaturalización de las proteínas, consistente en la alteración de su estructura tridimensional, sin ruptura de enlaces peptídicos. Ello conlleva una reducción de su solubilidad y accesibilidad con la consiguiente reducción de su degradación en el rumen (Blanchart, 1988). En esta reducción interviene la formación de enlaces entre los grupos aldehídos de los azúcares y los grupos aminos libres de la proteína. Sin embargo, si el calentamiento es excesivo se producen reacciones de Maillard o de amarronamiento no enzimático que implican la degradación de los azúcares a compuestos fenólicos, la condensación de estos con los aminoácidos y su posterior polimerización (Van Soest, 1994), siendo los compuestos resultantes indigestibles.

El principal desafío es, pues, identificar condiciones de tratamiento que incrementen la proteína no degradable digestible, a un grado que justifique el coste del tratamiento y con una mínima perdida en la disponibilidad de aminoácidos (NRC, 2001). De forma práctica, tradicionalmente, la proporción de nitrógeno insoluble en ácido detergente (ADIN) ha sido usada como un indicador del daño por calor en las proteínas (Goering y col., 1972; Thomas y col., 1982).

Las condiciones de tiempo, humedad y temperatura que proporcionarán una protección óptima resultan variables en función del suplemento a proteger. Sin embargo, el efecto de tratamientos moderados con calor en la degradación de la proteína no ha sido consistente. Así, Tagari y col. (1986), calentando harina de soja a 140 ºC o más, redujeron la liberación de amoniaco *in vitro*, mientras que el calentamiento a 120 ºC no produjo efectos. De forma similar, Mir y col. (1984) mostraron que el calentamiento a 110 o 120 ºC durante 120 o 20 minutos, respectivamente, redujo la degradación ruminal *in situ* de harina de colza pero no de la harina de soja.

Muchos tratamientos químicos han sido utilizados en el pasado con el objetivo de disminuir la degradabilidad de las proteínas. Sin embargo, algunos productos, como por ejemplo el formaldehido, han sido prohibidos por las directivas de la Unión Europea. El principal objetivo con el tratamiento de las proteínas con agentes químicos es crear una modificación reversible en éstas dependiente del pH, que permita inhibir su degradación en el compartimento rumen-retículo (donde el pH es cercano a la neutralidad o moderadamente ácido), pero no en el abomaso y el duodeno proximal donde el pH es mucho más bajo (Tamminga, 1979).

El tratamiento con ácidos desnaturaliza las proteínas (Waltz y Loerch, 1986), pudiendo ser los ácidos orgánicos e inorgánicos. Inicialmente, los estudios para disminuir la degradabilidad de los concentrados proteicos se realizaron con ácidos orgánicos

monocarboxílicos: fórmico, acético, propiónico, etc. (Vicini y col., 1983; Khorasani y col., 1989; Mckinnon y col., 1991), siendo limitada la protección obtenida y en ciertos casos no permanente, dado el carácter volátil de algunos de estos ácidos. Sin embargo, en épocas recientes existe un alto interés en el uso de ácidos di- o tricarboxílicos como alternativa a los antibióticos promotores del crecimiento en rumiantes, siendo el ácido málico el más utilizado entre ellos. La principal ventaja para el uso del ácido málico en el tratamiento de proteínas es su alta solubilidad en agua, y sus inconvenientes principales son su coste elevado y su alto poder de corrosión.

Dentro de los ácidos inorgánicos, solamente el ácido ortofosfórico está autorizado para su uso en piensos de rumiantes, ya que el uso de los ácidos clorhídrico y sulfúrico solamente está permitido en ensilados. El ácido ortofosfórico es líquido en estado puro, corrosivo, palatable a dosis baja, produce poco olor y, además, es más económico que los ácidos orgánicos (Mateos y col., 1999). Estas características le convierten en un potencial agente protector.

Ouarti y col. (2006) indicaron que la combinación del tratamiento térmico junto con el empleo de ácidos podría permitir alcanzar niveles más altos de protección que usando cada método por separado, y presentar ventajas económicas, debido a la disminución en el coste energético del tratamiento térmico y a la menor dosis de ácidos necesaria, y la menor posibilidad de generar reacciones de Maillard irreversibles, asociadas con la sobreprotección de proteínas.

Los tratamientos combinados de concentrados proteicos vegetales han dado resultados positivos. Wright y col. (2005) no observaron diferencias en la degradación ruminal de la proteína de harina de colza sin tratar o tratada con calor a una temperatura de 100 ºC durante 120 minutos, sin embargo cuando al tratamiento por calentamiento se le añadió un 5% de lignosulfato, la degradación ruminal se redujo drásticamente de 71,5% a 29,9%. Además, las vacas en lactación que fueron alimentadas con la colza tratada con calor y lignosulfato

excretaron menos N en la orina (como proporción de N consumido) y presentaron menores concentraciones de NH_3 ruminal y de urea en sangre y en leche que las vacas alimentadas con la harina de colza no tratada. Arroyo y col. (2013) también aumentaron en un 267% el contenido de PNDR de harina de girasol tratada con una solución de ácido málico (1 M) o ácido ortofosfórico (0,7 M) combinado con calentamiento a 150 ºC durante 6 h, independientemente del ácido usado. Como consecuencia de estos cambios, la eficacia de digestibilidad intestinal efectiva de la proteína de este concentrado aumentó en un 11,8% (ác. ortofosfórico) y un 20% (ác. málico). Por el contrario, Ouarti y col. (2006), utilizando un tratamiento en base a ácido málico y calor, no modificaron significativamente la degradabalidad ruminal ni la digestibilidad intestinal de una harina de soja, si bien la degradabilidad inicial de ésta ya era baja.

1.6. Concentrados de proteína vegetal producidos en España

La harina de girasol es el principal concentrado proteico producido en España; así, se cultivan más de 865.000 ha, con una producción de más de 1.000.000 t de semilla de las que se obtiene como subproductos de molturación 576.000 t de harinas de girasol (Anuario de Estadística, 2014). La proteína del girasol es deficitaria en lisina, pero rica en aminoácidos azufrados y triptófano, por lo que se complementa bien con la proteína de leguminosas (FEDNA, 2003). Entre éstas, el guisante es deficitario en aminoácidos azufrados y triptófano pero presenta una elevada concentración de lisina, siendo, además la principal semilla de leguminosa que se produce en España (200.000 t, Anuario de Estadística, 2014). Otra característica de gran interés es su alta concentración en almidón (rico en amilopectina) de lenta fermentación en el rumen, lo que unido a un contenido moderado en fibra digestible resulta útil para disminuir los riesgos de acidosis (FEDNA, 2003).

El principal problema de ambos concentrados proteicos es la elevada degradabilidad de su proteína en el rumen. Para la harina de girasol puede ser superior al 80% (González y col.,

1999; Woods y col., 2003; Rotger y col., 2006; Arroyo y col., 2013) y para el guisante puede sobrepasar el 90% (Poncet y Rémond, 2002; FEDNA, 2003). Ambos concentrados son pues claros candidatos a la protección de sus proteínas, pudiéndose teóricamente esperar a partir de la complementariedad de sus perfiles de aminoácidos esenciales una adecuada utilización metabólica de los aminoácidos absorbidos resultantes de la digestión intestinal de la PNDR resultante.

CAPITULO 2

OBJETIVOS

El objetivo principal de este proyecto de tesis fue incrementar la eficacia de utilización proteica de las dietas de rumiantes mediante el empleo de proteínas protegidas y la mejora en la predicción del aporte de proteína microbiana. Los objetivos específicos del trabajo fueron:

1. Reducir la degradabilidad ruminal de harinas de girasol y guisante mediante su desnaturalización mediante tratamientos combinados de pulverización con soluciones ácidas (ácidos ortofosfórico o málico) y posterior aplicación de calor.
2. Determinar los efectos de estos tratamientos en la degradación ruminal y la digestión intestinal de la proteína en estas harinas protegidas.
3. Mejorar la estimación de la contribución de la proteína microbiana al flujo de proteína by-pass.
4. Estudiar la respuesta productiva y/o la calidad de las producciones de ovinos de engorde, así como el posible ahorro de proteína en sus dietas, por la utilización en éstas de proteínas protegidas.

CAPITULO 3

SUNFLOWER MEAL AND SPRING PEA RUMINAL DEGRADATION PROTECTION USING MALIC ACID OR ORTHOPHOSPHORIC ACID-HEAT TREATMENTS

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Abstract

The effects of solutions of malic or orthophosphoric acids (0.752 Eqg/kg of feed) and heat to protect proteins of sunflower meal (SFM) and spring pea (SP) against ruminal degradation were studied using particle transit, ^{15}N infusion, *in situ* and electrophoretic techniques. Three wethers fitted with rumen and duodenum cannulae were successively fed three isoproteic diets including SFM and SP, untreated or treated with malic (MT) or orthophosphoric (PT) acids. Incubations of tested meals were only performed while feeding the respective diet. Estimates of the ruminally undegraded fraction (RU) and its intestinal digestibility (ID) of dry matter (DM), organic matter (OM, only for RU), crude protein (CP) and starch (only in SP) were obtained considering ruminal microbial contamination and particle comminution and outflow rates. When corrected for microbial contamination, estimates of RU and ID decreased in all tested fractions for both feeds. All RU estimates increased with the protective treatments, whereas ID-DM also increased in SFM. Low ID-CP values suggested the presence of antitrypsin factors in SP. Protective treatments of both feeds led to consistent increases in the intestinal digested fraction of DM and CP, being only numerically different for SP-starch (60.5% as average). However, treatments also reduced the OM fermentation which may decreaseruminal microbial protein synthesis. Electrophoretic studies showed albumin disappearance in both SFM and SP, whereas changes in other RU proteins were more pronounced in SP than SFM.

Keywords: protein protection; malic and orthophosphoric acids; heat; sunflower meal; spring pea.

Introduction

Applying protective treatments against ruminal fermentation in high quality proteins is attractive to avoid their microbial degradation, which is usually associated with high ruminal ammonia losses and also with reduced efficiency of microbial protein synthesis (NRC 2001). The inefficiency associated with excessive ruminal protein degradation is important in productive ruminants, whose large amino acid requirements should be supplied mainly by protein concentrates. In particular, this inefficiency is large for concentrates composed of highly degradable proteins such as sunflower or spring pea meals (SFM and SP meals, respectively). In addition, amino acids from these protein concentrates are transformed into compounds such as nucleic bases or amino sugars that are not used for protein synthesis in animal tissues. In SFM, Arroyo *et al.* (2011, 2013) reported a large protection of CP by treating it with malic or ortophosphoric acid solutions and heat. These treatments increased bypass CP by 4.25 times, but the undigested fraction was increased as well, especially when using orthophosphoric acid. This protective effect was suspected to be the result of the high temperature applied (150ºC during 6 h), combined with the high moisture from the acid solution (400 ml/kg). The objective of this study was to determine the effects of these treatments applied at lower temperature and moisture on rumen degradation and intestinal digestion of SFM and SP.

Materials and methods

Meal treatments

Samples of SFM and SP were treated with 4 N solutions (200 ml/kg) of malic acid (268.2 g/l) or orthophosphoric acid (130.6 g/l). Acid doses were equivalent to those (2 N solutions sprayed at 400 ml/kg) used by Arroyo *et al.* (2013). Two fractions of 12.5 kg of each meal were successively sprayed with each acid solution in a concrete mixer using a sprayer.

Both fractions were then mixed and allowed to rest for one hour at room temperature. The blend was then dried in a forced air oven at 120ºC for one hour. Then the oven was turned off and the treated material was left in the oven overnight. During the drying process, the material was stirred every 30 min during the first two hours and then every 60 min for the subsequent 5 h.

Diet and animals

Diets were isoproteic and included 45% oat (*avena sativa)* hay and 55% concentrate (fresh weight). The concentrate contained corn grain (30%), barley grain (30%), SFM (15%), SP (22%), and minerals and vitamins (3%). The control diet (UD) included untreated SFM and SP, which were replaced by equivalent amounts of meals treated with malic or orthophosphoric acids in diets including treated meals (MD and PD, respectively). The CP supplied from SFM and SP represented about 55% of total CP in all concentrates. Chemical composition of oat hay, SFM and SP meals and concentrates is shown in Table 1. Ranges of CP, neutral (NDF) and acid (ADF) detergent fibres of diets were (g/kg of DM): 177-181, 323-326 and 150-154, respectively. The DM intake was fixed at 45 g/kg $BW^{0.75}$ and diets were distributed in six equal meals (every 4 h), starting at 0900 hours. The diets UD, PD and MD were offered successively in three experimental periods to three wethers (BW = 57.4 ± 5.29 kg) fitted with rumen cannulae (inside diameter 60 mm) and T-type cannulae (inside diameter 12 mm) in the proximal duodenum. Wethers were pen-housed individually and handled according to animal care principles published in the Spanish Royal Decree 1201/2005 (BOE 2005).

Table 1. Chemical composition (g/kg of dry matter) of untreated (UT), malic acid treated (MT) and orthophosphoric acid treated (PT) of sunflower and spring pea meals, oat hay and used diets.

	Sunflowermeal			Spring pea			Hay	Diets		
Item[A]	UT	MT	PT	UT	MT	PT		UD	MD	PD
OM	932	923	889	963	969	933	840	885	880	881
Starch	-	-	-	368	367	370	-	-	-	-
NDF	309	311	261	201	245	224	459	323	326	324
ADF	177	184	159	85.3	94.5	103	236	154	150	151
Lignin	28.2	44.7	32.5	2.44	1.79	2.38	46.6	26.7	26.9	25.4
CP	344	322	346	221	210	212	189	181	177	178
NDIN (g/kg N)	137	151	103	125	180	150	358	-	-	-
ADIN (g/kg N)	41.9	47.3	38.2	18.8	21.9	23.0	54.2	-	-	-

[A]OM: organic matter; NDF: neutral detergent fibre; ADF: acid detergent fibre; CP: crude protein; NDIN and ADIN: insoluble nitrogen in neutral and acid detergent solutions, respectively.

Experimental procedures

Ruminal digestion

The experimental period of 21 days included successively: 10 days of diet adaptation, SFM and SP particle transit study (days 11 to 14) and ruminal nylon-bag incubations (days 15 to 21). The rumen was emptied at 0900 h at the end of *in situ* incubations in each period to isolate solid adherent bacteria (SAB) as described by Rodríguez *et al.* (2000). Rumen bacteria were labelled from day 11 to 21 by continuous infusion (250 ml/d) of a $(^{15}NH_4)_2SO_4$ solution (98 atoms% enriched) that provided 25 mg ^{15}N/d/wether.

The study of particle transit in the rumen-reticulum was performed by pulse dosing each animal before the first morning meal (i.e. at 0900 hours). Samples of 50 g of untreated SFM and SP were labelled with europium (Eu) and ytterbium (Yb), respectively, and were consumed completely in 20 min. These meals had previously been washed in an automatic washer to eliminate soluble components, and marked by immersion in solutions of Eu_2Cl_3 or $YbCl_3$ (10 mg of Eu or Yb/g of feed) as described by González *et al.* (1998). A total of 22 samples were obtained through the duodenal cannulae, the first sample before supplying the marker and the remaining samples between 1.5 and 96 h post marker dosing. Samples were oven dried at 105ºC for 48 h, milled to pass a 1 mm screen, and analysed for Eu and Yb. The pattern of Eu or Yb concentrations in the duodenal digesta over time was described for each animal by fitting to the model of Dhanoa *et al.* (1985). Primary and secondary rate constants used in this model were assumed as the rates of outflow (k_p) and of comminution and mixing (k_c) of particles in the reticulo-rumen, respectively, in agreement with Ellis *et al.* (1979) and González *et al.* (2006).

Ruminal incubations were performed using nylon bags (11 by 7 cm internal dimensions) with 46 μm pore size and filled with ~3 g (air-dry basis) of sample (ground to

pass a 2 mm screen). Two series of ruminal incubations with 14 bags containing SFM (7) or SP (7) meals were carried out in each diet at 2, 4, 8, 16, 24, 48 and 72 h. The meals incubated in each experimental period were those corresponding to the associated diet. In each incubation, all bags were placed simultaneously in the rumen just before the wethers were offered their first morning meal. Once collected from the rumen, bags were washed with tap water and stored at -20ºC. After thawing, bags were washed three times for 5 min in a turbine washing machine (Jata 580; JATA, Abadiano, Bizkaia, Spain). The same washing procedure was applied for two series of two bags for each meal to obtain the 0 hours value. Bags were stored at –20ºC once again, freeze-dried and immediately weighed to establish degradation kinetics of DM with the exponential model of Ørskov and McDonald (1979):

$$d = a + b\,(1 - e^{-kdt})$$

In this model, the constants *a* and *b* represent, respectively, the soluble fraction (which was assumed as the 0 hours value) and the nonsoluble but degradable component, which disappears at a constant fractional rate, $k_{d,}$ per unit time. The undegradable fraction (*r*) was estimated as $1 – (a + b)$.

Values of ruminally undegraded (RU) DM, organic matter (OM), CP and starch (only in SP) non-corrected or corrected for microbial contamination were determined considering degradation kinetics and both k_p and k_c transit rates with the method proposed by Arroyo and González (2013). This method is based on generating composite samples (CS) representative of the chemical composition of the ruminally undegraded feed. For this purpose, the residues obtained at each incubation time were pooled in equal quantities for each animal. The resultant residues for 0, 2, 4, 8, 16, 24, 48 and 72 h of incubation were considered representative of the composition of the rumen outflow of undegraded feed in the intervals 0 to 1, 1 to 3, 3 to 6, 6 to 12, 12 to 20, 20 to 36, 36 to 60 and 60 to 84 h, respectively. The proportions in which the different residues were mixed were calculated by the ratio of the flow in each interval and the

total flow determined using the feed flow-function proposed by Arroyo and González (2013). Composite samples were analysed for OM, CP, ^{15}N/total N and starch (only in SP). The respective RU values (as %) were determined from the concentrations of the tested fraction in the composite samples (Y) and in the whole feed (X) and the RU-DM values, as follows:

RU-MO, -CP or -starch = Y × RU-DM/X

The microbial proportions of N and DM in CS were determined as indicated by Rodríguez and González (2006) using SAB samples as reference; SAB isolates were lyophilised and analysed for DM, OM, N, ^{15}N/N and total glucose. Microbial proportions of OM and glucose were determined as the microbial DM content of CS samples × the concentration of these fractions in SAB expressed on DM.

Intestinal digestion

Twenty days after the end of the ruminal studies, wethers were fed again the untreated diet to determine ID-DM, -CP and -starch of untreated and treated meals. Eight sub-samples of ~200 mg of each composite sample were put into round-shaped (approximate diameter 2.5 cm) mobile nylon bags. These bags were inserted randomly through the duodenal cannulae of the respective wether, at a rate of one bag every 15 min for 2 h for a total of 8 bags/d/wether, and recovered from the faeces. Bags were then processed by the same methods as those for rumen incubations; bags were then dried at 80ºC for 48 h and weighed. The ID-DM was calculated as DM disappearance from the bag during intestinal incubation. Undigested residues obtained in each wether were pooled and analysed for N, ^{15}N/N and starch (only in SP). The ID for any fraction was determined as the proportion from the ID-DM value and concentration of this fraction in the composite sample (Y) and in the intestinal incubated residues (Z):

ID-CP or -starch = 1 - [Z × (1 –ID-DM)/Y]

The $^{15}N/N$ ratio was used to correct for the residual contamination due to adherent rumen microorganisms as previously indicated.

Protein electrophoresis

Proteins in untreated and treated meals and in their 0 h, composite and intestinally undigested samples were extracted and subjected to electrophoresis to determine the digestion site of the different protein fractions.

Chemical and protein electrophoresis analyses

Feed samples were analysed in triplicate using AOAC (2000) procedures for DM (procedure 934.01), ash (procedure 967.05) and CP (6.25 x Dumas N; procedure 968.06), NDF (Van Soest *et al.* 1991) and sequential ADF and acid detergent lignin (Robertson and Van Soest 1981). Analyses of NDF were performed with alpha-amylase and without sodium sulphite. NDF and ADF were expressed including the residual ash. The insoluble nitrogen in neutral (NDIN) and acid (ADIN) detergent solutions was determined by N analysis of the NDF and ADF residues, respectively. Ruminal or intestinal incubated residues were also analysed for N with the Dumas method. Samples of duodenal contents collected for transit studies were analysed by atomic absorption (Yb) or emission (Eu) spectrometry, as described by González *et al.* (1998). Nitrogen isotopic proportions were performed in an elementary analyser (Flash 1,112, Thermo, Bremen, Germany) coupled in continuous flow to an isotope ratio mass spectrometer (Delta V, Thermo, Bremen, Germany).

Homogenate samples of the different protein fractions of SFM and SP were extracted using different solutions: (1) Tris-HCl pH 6.8 1 M, 10% glycerol, 2.5% SDS and 5% β-mercaptoethanol to obtain total protein (2) 0.4 M NaCl + 3% β-mercaptoethanol to obtain globulins and (3) water to obtain albumins. Solutions were used at 750 µl per mg of nitrogen. The extracts were agitated in a vortex and left overnight with gentle stirring at 4°C. After

centrifugation (12,500 rpm for 45 min at 4ºC) and pellet removal, the supernatant was mixed with a reducing buffer (1:1, v/v) and boiled for 10 min in a water bath. Electrophoresis was carried out using polyacrylamide slab gels (SDS-PAGE; 160 x 155 x 1 mm) following the Laemmli (1970) protocol modified by Sánchez-Yélamo (in Vázquez *et al.* 2000). For total proteins and globulins, 10 µl per sample were applied, whereas for albumins, 20, 30 and 40 µl per well were tested because fewer proteins were detected in the gels. In all cases the electrode buffer was cold Tris-glycine (pH 8.3) and bromophenol blue was used as front-dye marker. The molecular weights of the protein samples were determined using a standard protein marker (Invitrogen Mark 12) consisting of myosin (MW 200 KD), β-galactosidase (MW 116.3 KD), phosphorylase b (MW 97.4 KD), serum albumin (MW 66.3), glutamic dehydrogenase (MW 55.4 KD), lactate dehydrogenase (MW 36.5 KD), carbonic anhydrase (MW 31.0 KD), trypsine inhibitor (MW 21.5 KD), lysozyme (MW 14.4 KD) and aprotinin (MW 6.0 KD). After electrophoresis (about 4.5 h), gels were stained overnight at room temperature with Coomassie Brilliant blue R-250 followed by de-staining twice in acetic acid: 2-propanol 10% (v/v) for 4 hours. At least three repetitions were made to check for the reproducibility of the electrophoretic patterns. In each band pattern, bands were numbered in ascending order from cathode to anode starting with band "1". The identity of each band was estimated by its relative mobility (Rm) on the gel, and the molecular masses were estimated using a calibration curve developed with the marker standard polypeptides. Several specific proteins were identified by comparison with data from the literature.

Statistical methods

Degradation and transit kinetics were fitted by non-linear regression. Effects of protective treatments (*t*) on apparent parameters of DM degradation kinetics and RU-DM were studied by variance analysis considering these factors and the wethers (*w*) in the model ($y_{ij} = \mu + t_i + w_j + \varepsilon_{ij}$). These same effects and those of the correction of microbial contamination taking

place in the rumen (*c*) of RU, ID and the intestinal absorbable dietary fraction (IADF = RU×ID) values were studied by variance analyses in a split-plot arrangement of treatments. In this design, the treatment was the whole-plot, tested against the wether × treatment interaction as the error term, and microbial correction and its interaction with treatment were the sub-plot treatments ($y_{ijk} = \mu + t_i + w_j + c_k + t_i \times c_k + t_i \times w_j + \varepsilon_{ijk}$). These means were examined by orthogonal contrasts for treatment effects (untreated vs treated) and of acid used (malic vs orthophosphoric acids). This same design substituting microbial correction by the transit model considered (k_p alone compared to k_p and k_c) was also used to examine the differences in RU estimates of the insoluble but potentially degradable fraction of DM. As the animals were adult, maintained in a controlled environment, the period effect was assumed to be negligible in all variance analysis. Effects were declared significant at $P<0.05$. All statistical analyses were performed using SAS software, version 8.0 (SAS 1999).

Results

Feed digestion

There were no differences among treatments or between the two meals (SFM, SP) in the k_p or k_c values: k_p: 6.36%/h vs 5.53%/h; s.e.m. = 0.295; $P = 0.227$; k_c: 77.7%/h vs 56.2%/h; s.e.m. = 7.05; $P = 0.153$. Therefore, mean values for each feed were used to calculate RU values in untreated and treated meals.

In treated SFM and SP, the soluble fraction of DM (*a*) decreased and the potentially degradable fraction (*b*) increased; however, the undegradable fraction (*r*) was increased except when SFM was treated with orthophosphoric acid (Table 2). In addition, both treatments led to reductions of k_d in SFM while reductions in k_d for SP were only numerical. As a result of these changes, treatments increased the apparent RU-DM corresponding to both the b fraction and total feed in both SFM and SP. There was no clear evidence of differences in the

Table 2. Effects of protective treatments on apparent rumen degradation kinetics and ruminal undegraded fraction (RU) of dry matter of sunflower and spring pea meals (values are expressed as %).

	Meals			Probability of contrasts[B]		
	UT[A]	MT	PT	s.e.m.	C1	C2
Sunflower meal						
a[C]	31.6	26.3	26.2	-	-	-
b	50.2	52.0	55.6	4.65	0.003	0.006
r	18.3	21.6	18.2	4.65	0.045	0.006
k_d (%/h)	14.0	8.64	9.94	1.13	0.027	0.464
RU	32.2	41.6	37.9	0.72	0.001	0.021
RU-*b*	13.9	20.0	19.7	1.03	0.009	0.844
Spring pea						
a	38.9	23.2	21.6	-	-	-
b	55.8	68.2	68.8	1.27	0.001	0.764
r	5.29	8.56	9.60	1.27	0.071	0.592
k_d(%/h)	11.7	8.95	10.5	0.92	0.153	0.311
RU	20.6	31.4	30.2	1.04	0.001	0.427
RU-*b*	15.3	22.9	20.6	1.38	0.019	0.300

[A]UT, MT and PT: meals untreated or treated with malic or ortophosphoric acids, respectively.
[B]C1: UT vs treated meals; C2: MT vs PT.
[C]*a*, *b*, and *r* represent soluble, non-soluble degradable and undegradable fractions, respectively. k_d: fractional degradation rate of fraction *b*. RU: ruminally undegraded fraction. RU-*b*: RU corresponding to the *b* fraction, calculated using ruminal rates of particle comminution (k_c) and outflow (k_p) as:RU = *r* + RU-*b* and RU-*b* = $bk_ck_p/((k_d+k_p)(k_d+k_c))$

protective effect between both acids, especially for SP. The omission of k_c from the model resulted in mean values (± s.e.) of the RU-DM of the *b* fraction for UT, MT and PT meals of: 16.2 (±2.14), 22.2 (±1.19) and 22.1 (±1.67) % in SFM and 18.4 (±1.48), 26.5 (±1.86) and 24.3 (±1.80) % in SP, respectively. These values were higher ($P < 0.001$) than those generated when k_p and k_c were included in the calculations which are indicated in Table 2. Therefore, omitting

k_c overestimated the apparent RU-*b* of DM of UT, MT and PT meals respectively by 16.8, 11.0 and 12.4% in SFM, and by 20.1, 15.7 and 18.2% in SP (data not shown).

The microbial contamination with DM or N in CS samples of both feeds showed large variability, and treatment effects were undetected. The large variability observed for the disappearance of DM biomass during the intestinal incubation in SP also prevented detecting effects which on the contrary were shown for SFM (Table 3).

Table 3. Microbial contamination of ruminal undegraded composite samples of sunflower meal (SFM) and spring pea (SP).

	Meals			Contrast probability[B]		
	UT[A]	MT	PT	s.e.m.	C1	C2
DM (g/kg residual DM)						
SFM	39.4	30.5	46.3	6.99	0.91	0.185
SP	30.9	25	25.5	3.8	0.295	0.924
N (g/kg residual N)						
SFM	69.8	37.8	61.8	10.72	0.202	0.19
SP	91	53.2	56.2	13.46	0.093	0.882
Intestinal disappearance of ruminal biomass (% of DM)						
SFM	86.9	77.8	88.4	0.69	0.011	<0.001
SP	65.9	85.6	77.8	11.09	0.307	0.645

[A]UT, MT and PT: meals untreated or treated with malic or ortophosphoric acids, respectively.
[B]C1: UT vs treated meals; C2: MT vs PT.

Not correcting ruminal microbial contamination led to consistent overestimations in RU, ID and IADF values of all tested variables in both tested feeds (Tables 4 and 5). As previously indicated for DM, protective treatments consistently increased RU in the remainder tested fractions in both feeds. Also, positive effects were shown in the ID-DM in SFM, but not for ID-CP in this meal or for the different SP tested fractions. As a result, treatments also increased the IADF-DM and IADF-CP in both tested meals (Tables 4 and 5). Despite the fact that treatments increased the IADF-starch by 60.5% on average, the results were not statistically significant, probably due to the high variability observed (Table 5).

Regression analysis showed strong correlations between microbial corrected values of both RU-DM and ID-DM in SFM (ID-DM = -0.55 + 0.955 RU-DM; n = 9; R^2 = 0.654; *P* = 0.008). Similar relationships were also shown between IADF and RU of all tested parameters in both feeds (Figure 1). In SFM, malic acid showed higher protective efficiency than orthophosphoric acid for RU values as well as for IADF-CP (Table 4); this was not observed for SP (Table 5).

Protein electrophoresis

Gel electrophoresis of total proteins, globulins and albumins are shown in Figures 2 (SFM) and 3 (SP). In both feeds, images of treated meals were less sharp than those observed in untreated meals, especially in those samples not subjected to washing processes prior to protein extraction and electrophoresis. Profiles of SFM showed clear bands for polypeptides in an approximate range of molecular weights (MW) of 56 to 6 KD, whereas those of SP fell within a range of 97.4 to 6 KD.

Albumins were not detected in 0 h samples of SFM nor in those of protected SP meals. In addition, they were not detected in both feeds, untreated or treated CS samples. Similarly, the gel of total proteins of CS samples showed lack of some polypeptides other than albumins in untreated or treated SFM (3, 13 and 14) and SP (1, 2, 4, 6, 12, 14 and 21). Furthermore, some polypeptides (10, 13 and 20) were not detected in the gel of globulins in untreated or treated CS samples of SP. Finally, polypeptides were not observed in intestinal incubated residues of SFM or SP, with the exception of some diffuse shading in areas corresponding to polypeptides of low MW (<10 KD) identified as 15, 16 and 18.

Table 4. Effects of protective treatments and of correcting the microbial contamination taking place in the rumen on in situ estimates of ruminally undegraded fraction (RU), intestinal digestibility (ID) and intestinal absorbable dietary fraction (IADF: RU×ID) of sunflower meal.

	Meals[A]						Effects and contrasts[B]				
	UT		MT		PT		Treatments			Correction	
	NC[C]	C	NC	C	NC	C	s.e.m.	C1	C2	s.e.m.	*P*
RU											
Dry matter	32.2	30.9	41.6	40.3	37.9	36.1	0.61	< 0.001	0.010	0.17	< 0.001
Organic matter	32.8	31.8	43.4	42.3	40.5	39.0	0.61	< 0.001	0.022	0.14	< 0.001
Crude protein	21.4	19.9	42.2	40.6	33.0	30.9	0.61	< 0.001	< 0.001	0.19	< 0.001
ID											
Dry matter	30.0	27.7	38.1	35.8	40.1	37.4	2.07	0.024	0.573	0.38	0.004
Crude protein	80.1	75.0	83.4	79.5	85.4	78.0	2.92	0.320	0.952	0.87	0.004
IADF											
Dry matter	9.71	8.58	16.0	14.5	15.3	13.6	1.15	0.012	0.628	0.24	0.005
Crude protein	17.5	15.2	35.5	32.5	28.6	24.3	1.24	< 0.001	0.013	0.51	0.004

[A]UT, MT and PT: meals untreated or treated with malic or ortophosphoric acids, respectively.
[B]C1: UT vs treated meals; C2: MT vs PT.
[C]NC and C: no corrected and corrected by the ruminal microbial contamination.

Table 5. Effects of protective treatments and of correcting the microbial contamination taking place in the rumen on in situ estimates of ruminally undegraded fraction (RU), intestinal digestibility (ID) and intestinal absorbable dietary fraction (IADF: RU×ID) of spring pea.

	Meals[A]						Effects and Contrasts[B]				
	UT		MT		PT		Treatments			Correction	
	NC[C]	C	NC	C	NC	C	s.e.m.	C1	C2	s.e.m.	*P*
RU											
Dry matter	20.6	20.0	31.4	30.7	30.2	29.4	1.06	0.001	0.449	0.09	0.002
Organic matter	21.0	20.5	31.9	31.3	31.4	30.8	1.04	0.001	0.749	0.07	0.001
Crude protein	13.0	11.9	28.2	26.7	26.1	24.7	1.14	<0.001	0.270	0.16	0.001
Starch	15.4	15.4	29.2	29.1	25.9	25.8	2.01	0.008	0.300	0.007	0.003
ID											
Dry matter	32.1	30.8	32.9	31.6	35.9	34.7	1.90	0.373	0.313	0.22	0.006
Crude protein	35.2	24.8	37.5	28.1	38.6	29.8	2.00	0.222	0.659	1.68	0.007
Starch	64.7	64.2	54.5	53.6	62.7	62.1	6.63	0.484	0.427	0.12	0.007
IADF											
Dry matter	6.64	6.17	10.3	9.67	10.8	10.2	0.66	0.009	0.597	0.09	0.003
Crude protein	4.57	2.99	10.6	7.47	10	7.43	0.70	0.004	0.782	0.37	0.003
Starch	10.1	9.97	16.0	15.7	16.5	16.3	2.93	0.164	0.890	0.03	0.005

[A]UT, MT and PT: meals untreated or treated with malic or ortophosphoric acids, respectively.
[B]C1: UT vs treated meals; C2: MT vs PT.
[C]NC and C: no corrected and corrected by the ruminal microbial contamination.

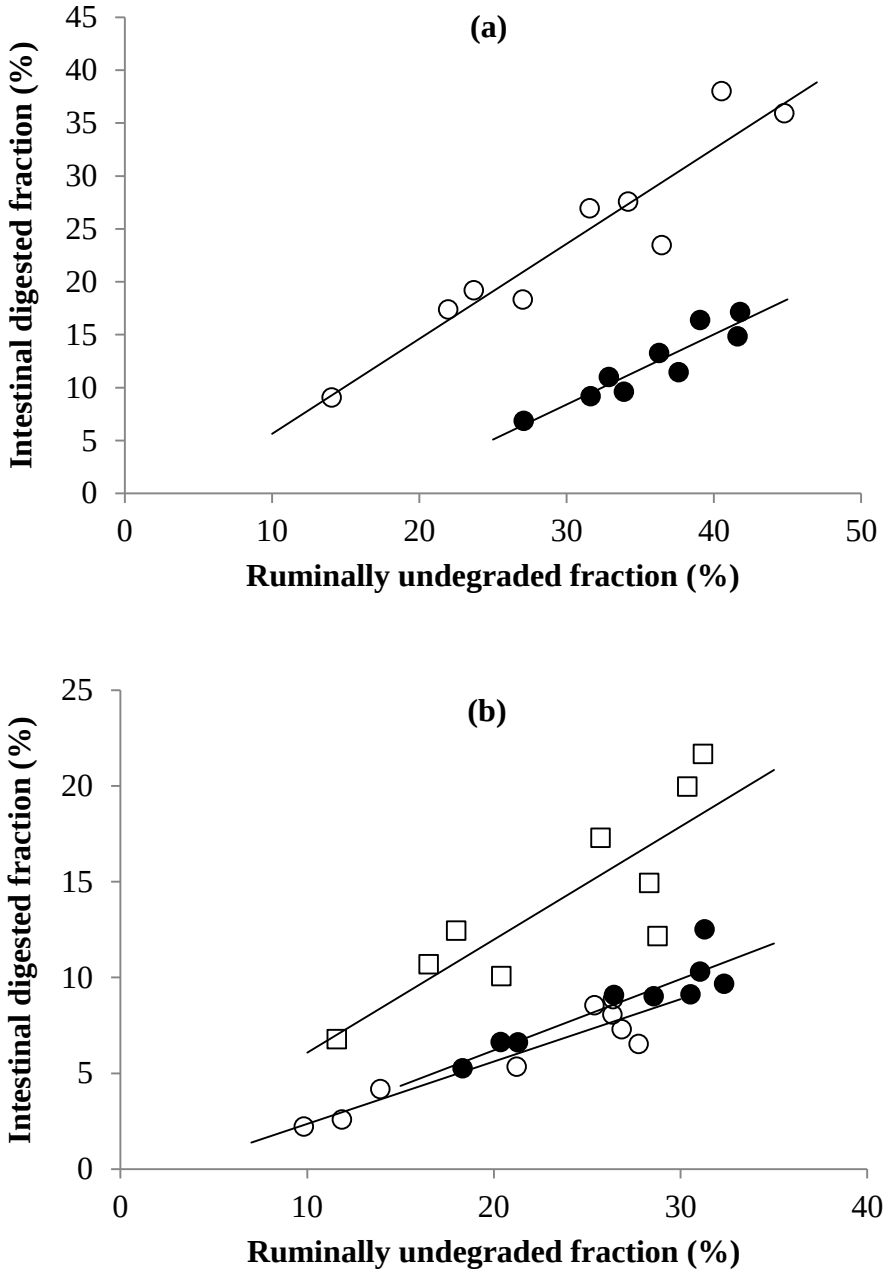

Fig. 1. Relationships between intestinal digested (IDF) and ruminally undegraded (RU) fractions of dry matter (●), crude protein (○) and starch (■) in sunflower meal (SFM; a) and spring pea (SP; b). Equations: SFM: DM: IDF = -11.47 +0.663 RU; R^2 = 0.861; P < 0.001. CP: IDF = -3.34 +0.898 RU; R^2 = 0.861; P < 0.001. SP: DM: IDF = -1.23 +0.371 RU; R^2 = 0.813; P < 0.001; CP: IDF = -0.90 +0.323 RU; R^2 = 0.867; P < 0.001; Starch: IDF = 0.17 +0.590 RU; R^2 = 0.861; P < 0.001.

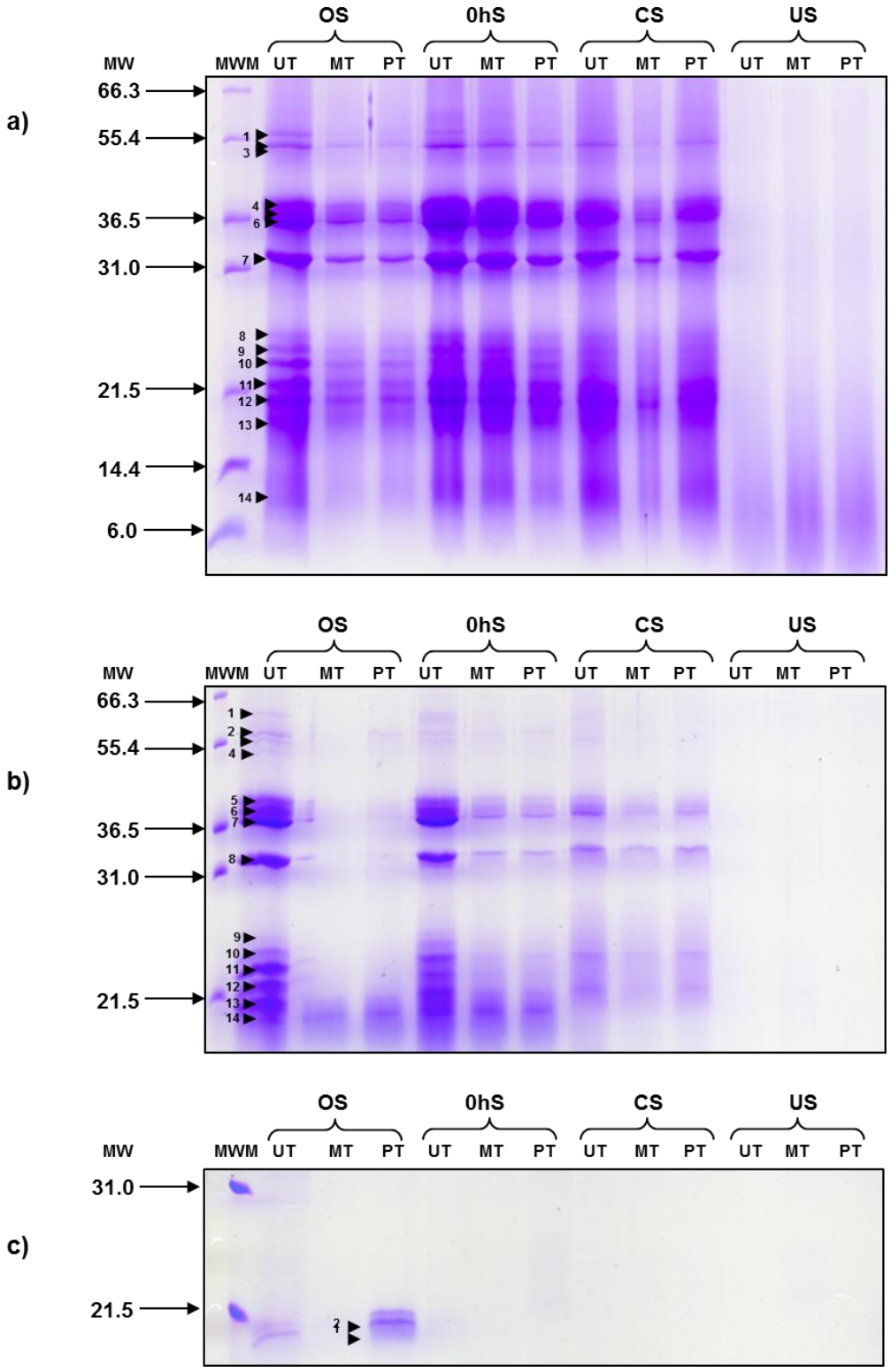

Fig 2. SDS-gel electrophoresis of total proteins (a), globulins (b) and albumins (c) of original (OS), 0 h (0hS), ruminally undegraded (CS) and intestinal undigested (US) samples of untreated (UT), malic acid treated (MT) and orthophosphoric acid treated (PT) sunflower meal.

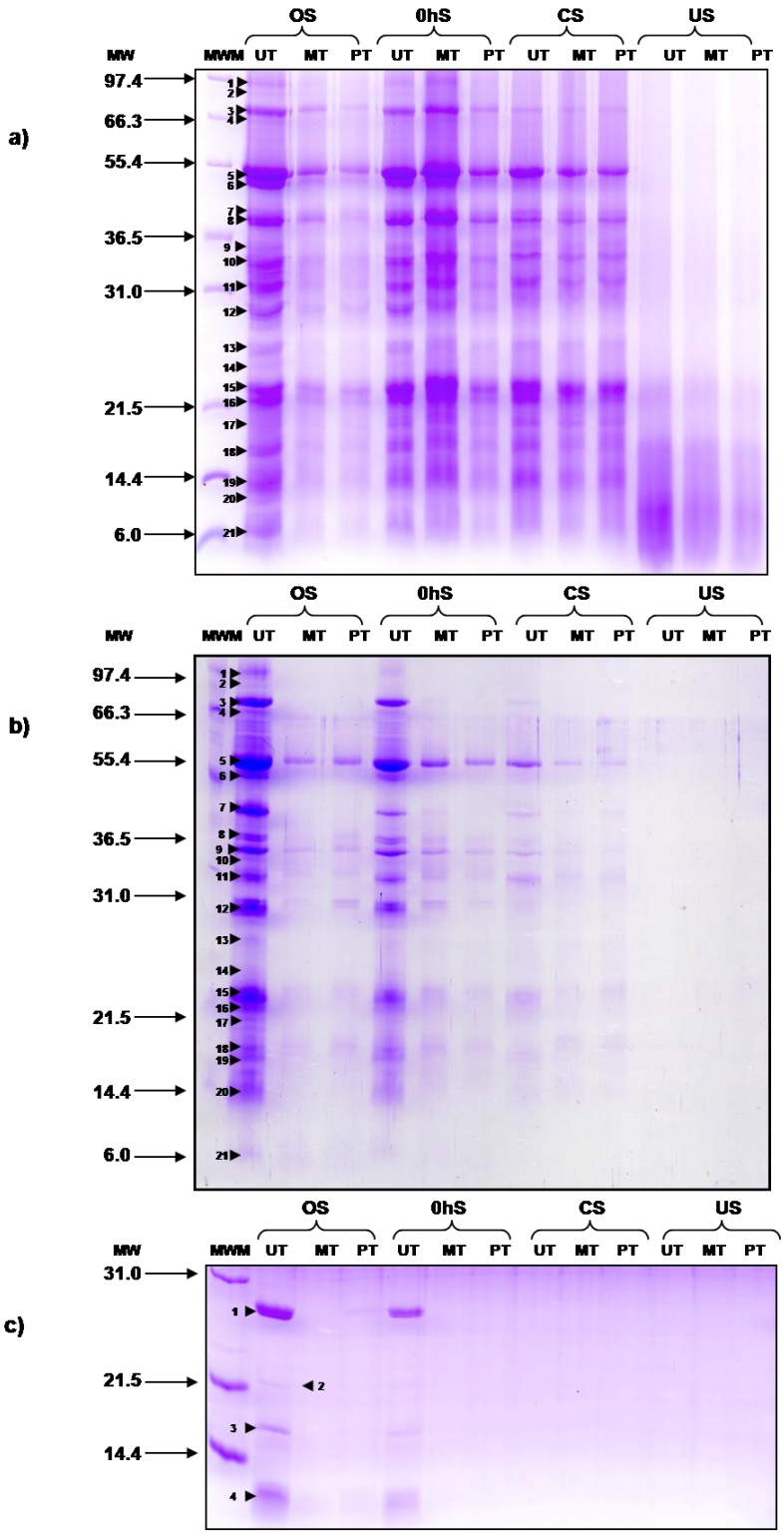

Fig 3. SDS-gel electrophoresis of total proteins (a), globulins (b) and albumins (c) of original (OS), 0 h (0hS), ruminally undegraded (CS) and intestinal undigested (US) samples of untreated (UT), malic acid treated (MT) and orthophosphoric acid treated (PT) spring pea.

Discussion

The chemical composition of the SFM sample may be considered normal except for its low fibre fractions in relation to its CP content. By contrast, the SP sample showed a high fibre content.

Accuracy of in situ estimates

The omission of the k_c rate from the calculations of in situ degradation may have an effect on the *in situ* digestive estimates through its influence on the digestion site (ARC 1984). In relation to the total mean residence time in the rumen-reticulum, the time associated with the k_c rate was considerable (7.6% and 9.0% as mean in SFM and SP, respectively) supporting the consideration of this rate to improve accuracy of *in situ* estimates, as previously indicated (Arroyo and González 2013). The overestimation of the feed bypass caused by the lack of correction of the microbial contamination of rumen-incubated residues may also lead to overestimations of ID due to the large intestinal digestion of these adherent microorganisms. Both overestimations are directly related to the extent of microbial contamination (Rodríguez and González, 2006), whereas the difference in intestinal disappearance between SAB and bypass compounds is also a major factor affecting ID overestimations (Arroyo and González 2013), which is in agreement with the large overestimations shown in ID-CP of SP. Current values for SFM showed higher contaminations (4.0 and 3.1 times as average of the three meals for the DM and CP, respectively) than those observed in another SFM sample of a previous similar experiment carried out with the same doses of both acids (Arroyo *et al.* 2013). As a consequence, RU and ID overestimations in the current sample were also largely higher.

Protective treatments

The reduction in degradability with the protective treatments in both tested meals was due to a decrease in the soluble fraction and increased *b* fractions, which in addition were

degraded more slowly. In consequence, treatments increased the *b*-RU fraction and, therefore, shifted the digestion site to the intestine. On the other hand, these effects were lower in SFM compared to previous results by Arroyo *et al.* (2013). The decrease in ruminal DM digestibility in SFM was mainly the result of decreased degradation of CP. Thus, microbial corrected values showed that the reduction in degraded CP represented 86.8% (with malic acid) and 70.1% (with orthophosphoric acid) of the reduction in ruminally digested DM. This was not the case in SP, which displayed respective values for this contribution of 35.8% and 31.6%, in agreement with its moderate CP content. Equivalent values for starch were 47.4% and 38.8%, respectively. These results show that degradation of components other than proteins is also affected by these treatments in both feeds.

Observed increases of RU-CP in treated SFM were lower than those reported in Arroyo *et al.* (2013). In addition, in the experiment aforementioned, associated increases of ID-CP were observed, whereas increases seen in the current experiment were not statistically significant. As a result the increments in microbial corrected values of IADF-CP (113.8% and 59.9% for malic and orthophosphoric acids treated meals, respectively) were also lower than in Arroyo *et al.* (2013). This lower protection efficiency may be associated with the lower CP degradability of the present untreated SFM sample, but mainly with the lower levels of moisture and heat applied in the present experiment. The similar behaviour observed with treatments in SP also support this limited protection efficiency. This fact was augmented in SP by its extremely low ID-CP, which reduces the benefits associated with changing the digestion site to the intestine. Thus, the contents of feed-undigested CP in the total tract were 8.88% in UT, 19.2% in MT and 17.2% in PT. The low ID-CP observed in SP cannot be associated with a high concentration of indigestible compounds in the RU fraction. Thus, low values were observed both in the untreated meal and in the treated meals which showed RU fractions 2.15 times higher on average than the untreated meal. On the contrary, it suggests the presence of a

non-heat labile antitrypsin factor in SP. Thus, the results of ruminal degradability of DM, CP and starch in the untreated SP sample were close to those proposed by the INRA (2007): 80%, 86% and 79%, respectively, whereas INRA (2007) suggests intestinal digestibility of CP of 91%; 2.5 times higher than the average values for untreated and treated SP meals reported in this study.

Changes with protective treatments in RU and ID demonstrate that the close relationships shown between IADF and RU for both feeds are mainly from the decrease in rumen degradation, although in SFM the increase of ID-DM also contributed to the increased IADF value. Differences in the regression coefficients among chemical fractions also show differential effects of these treatments, which were higher for CP in SFM and for starch in SP.

The increased supply of intestinally digested CP from treated meals should also be associated with a decrease in the ruminal microbial protein synthesis owing to their reduced OM degradation. Based on corrected results of IADF-CP and OM degradability (calculated as 100-RU) and the parameters of ruminal synthesis and intestinal availability of microbial protein in the PDI system (INRA 2007), the total supply of intestinal digestible protein from SFM samples was 111.7, 154.3 and 134.9 g/kg DM in UT, MT and PT meals, respectively. Similar values in SP were 78.0, 77.6 and 76.1 g/kg DM. When CP concentrations of the meals were considered, protective treatments using malic or orthophosphoric acids respectively increased the protein value by 47.5% and 20.1% in SFM and by 4.95% and 1.79% in SP. These increases may be somewhat higher than the above calculated values due to a higher efficiency of microbial synthesis associated with the reduction of the proportion of CP in the fermented OM (NRC 2001). The negative effect of these treatments on ruminal microbial synthesis through the reduction of fermented OM seems to indicate that they are mainly of interest in protein-rich feeds.

Results from the current study of SFM are in agreement with the conclusions of Arroyo *et al.* (2011, 2013) pointing to the higher efficacy of malic acid than orthophosphoric acid to protect proteins. In addition, the inclusion of malate in the diet may provide additional benefits for the ruminal fermentation pattern (Martin and Streeter 1995; Callaway and Martin 1996; Carro *et al.* 1999).

The range of MW in polypeptidic bands detected by SDS-PAGE is in agreement with that observed by Spencer *et al.* (1988) in both feeds. A proposition of band distribution comparing current MW values with literature data is presented in Table 6. An uncertain identification of some polypeptidic bands occurred in SFM, probably because the hexane extraction used in the industrial process may extract cell-wall proteins which are incorporated to the proteins present in the SDS-PAGE gels, hindering this identification. Albumin disappearance in 0 h samples of SFM agrees with its soluble character. On the other hand, residual albumins that showed in the 0 h sample of the UT-SP meal are in agreement with results of Spencer *et al.* (1988). These authors found albumins beyond 4 h of *in vitro* incubation, indicating that their degradation resistance was almost similar to that of bovine serum albumin, which is usually used as a standard of a protein relatively resistant to rumen degradation. However, the absence of albumin in CS profiles of untreated and treated meals of both feeds show that the practical contribution of albumins to ruminal protein outflow should be near zero even in SP. Bands of CS profiles in both feeds showed lower intensity compared with the original or 0 h samples, indicating quantitative changes resulting from degradation. The disappearance of polypeptidic bands in CS samples supports higher effects of ruminal degradation in SP than in SFM, which agrees with the quantitative CP degradation results. *In vitro* studies of Spencer *et al.* (1988) showed a faster breakdown of major subunits of convicilins, vicilins and α-legumins in peas. In addition, Aufrère *et al.* (1994 and 2001) indicated a faster *in situ* degradation of vicilins and convicilins than legumins in peas.

Present results provided partial support to these observations because most disappearing bands in SP have MW corresponding to both vicilins and convicilins. The higher degradation resistance of SP-legumins may be associated with the existence of disulphide bridges not present in the other SP-globulins (Casey and Domoney 1999). No polypeptidic bands were detected after intestinal digestion for SFM. Therefore, the undigested CP contents shown by *in situ* microbial-corrected data (4.70%, 8.10% and 6.6% in UT, MT and PT meals, respectively) should correspond mainly to non-protein compounds or too small peptides which cannot be detected with SDS-PAGE techniques. These kinds of compounds should also be present in intestinally digested SP-samples that also showed poorly resolved bands in the low MW areas, probably corresponding to non-digested peptides. The latter observation is in agreement with the low intestinal digestibility shown *in situ*.

Table 6. Range of estimated molecular mass of polypeptidic bands detected by SDS-PAGE in sunflower meal and spring pea studied samples.

Sunflower meal				Spring pea			
Band nº	Range of molecular mass (KD)	Protein nomenclature	References	Band nº	Range of molecular mass (KD)	Protein nomenclature	References
1	56	unknown		1 2	96-91	unknown	
2	53	unknown		3 4	70-68	Convicilin subunits	[1, 2, 3, 4, 5]
3	46	unknown		5 6	55-53	Vicilins (50 KD)	[2, 3, 4]
4 5 6	40-30	Helianthinin (larger polipeptides)	[2, 6, 3]	7 8	40-45	α Legumins (40KD)	[2, 3, 4]
7	33	unknown		9 10 11	36.5-31	Vicilins (30 KD)	[2, 3, 4]
8 9 10	27-23	Helianthinin (smaller polipeptides)	[2, 6,3]	12 13 14 15 16 17	30-20	β Legumins and vicilins	[2, 3, 4]
11 12 13	21.5-15	Albumins	[7]	18 19 20 21	20-6	Vicilins (of lower mol. mass) and albumins	[2, 3,4, 5]
14	12	2S-Methionine-rich Protein	[8, 5]				

[1] Croy et al (1980); [2] Derbyshire et al (1976); [3] Casey (1999); [4] Casey and Domoney (1999);

[5] O´Kane et al (2004); [5] Shewry and Pandya (1999); [6] Allen et al (1985); [7] Kortt and Caldwell (1990); [8] Kortt et al (1991).

Conclusions

The protective treatments tested in this experiment decreased the ruminal degradation, displacing the digestion site to the intestine. These effects were higher with malic than with orthophosphoric acid. However, a reduction of the moisture and heat compared to previous technical recommendations decreased the treatment efficacy. Also, the treatments' effectiveness decreased with protein concentration due to the reduction of the ruminal-fermented organic matter and, presumably, of the resulting microbial protein synthesis. On the other hand, their effectiveness is drastically reduced when anti-nutritive factors are present in the intestine, as seems to be the case of the SP sample tested. Correction for microbial contamination of ruminal-incubated residues was important for the accuracy of current estimates.

Acknowledgments

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CAPITULO 4

INFLUENCE OF THE BACTERIAL FRACTION USED AS REFERENCE ON ESTIMATES OF THE MICROBIAL NUTRIENT SUPPLY IN RUMINANTS

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Abstract

The chemical composition of bacteria associated with solid (SAB) and liquid (LAB) rumen-digesta phases was studied to examine the accuracy of a previous regression system determining the underevaluation of SAB-nutrient supply using ^{15}N as marker and LAB as microbial reference. Three rumen and duodenum cannulated wethers were feed in successive periods with three isoproteic diets including two protein concentrates (sunflower meal and spring pea) either untreated or treated with malic or ortophosphoric acids plus heat. Bacterial samples were isolated after 12 d of continuous infusion of $(^{15}NH_4)_2SO_4$ (25 mg ^{15}N/d). Compared with SAB, LAB showed lower contents of organic matter, polysaccharide-glucose and total lipids and the opposite for the crude protein content and the ^{15}N enrichment. Current data fitted well to the previous relationship predicting the ^{15}N enrichment of SAB from the same value in LAB. This new equation allows establishing the underevaluation in the supply of crude protein from the synthesized SAB in 22.0%. Another relationship calculated using mean diet values from the literature confirmed the magnitude of this error. This underevaluation was higher for the supply of glucose (43.1%) and still higher for the lipid supply (59.9%). These errors should be considered to improve ruminant nutrition. Predictions obtained using ^{15}N as marker are useful to correct the errors associated with the traditional use of LAB as reference, and, therefore, to obtain more accurate estimates of the microbial nutrient supply to the ruminants.

Keywords: rumen bacteria; chemical composition; ^{15}N enrichment; microbial nutrient supply.

Introduction

Microbes evacuated from the rumen largely contribute to the nutrients absorbed in the small intestine. Therefore, accurate estimations of the postruminal flow of microbial nutrients are basic to improve ruminant nutrition. However, for many years the microbial population usually used as reference to determine this supply has been bacteria harvested from the ruminal liquid fraction (liquid-associated bacteria; LAB), in spite that they are only a minor fraction of the rumen bacterial populations, as solid-associated bacteria (SAB) are largely predominant (Faichney 1980; Legay-Carmier and Bauchart 1989; Volden 1999; Rodriguez et al. 2003). On the other hand, it is well known that for the most usual microbial markers, such as diamino pimelic acid, nucleic acids or its derivatives as well as nitrogen or phosphorus isotopes, the marker/N ratio is higher in LAB than in SAB and as a consequence, the use of LAB as reference underestimates the postruminal microbial flow (González et al. 2012). Mixed bacterial samples (LAB + SAB) isolated from mixed liquid and solid digesta contents have been used as reference in some studies to avoid the formerly cited problem. However, there is no certainty that the proportions in these samples of both populations will be representative of the bacteria mix reaching the duodenum. Although there are numerous works comparing both populations of bacteria, the effects of using LAB as reference on nutrient supply estimates on ruminants are poorly defined. The magnitude of the errors in the SAB-nutrient supply using LAB as reference can be directly established from the marker/N ratios and the contents of the examined fraction in both bacterial populations (González et al. 2012). This last work points to a close relationship between the ^{15}N enrichments of both bacterial populations which may simplify these estimations. In the current work we have used the bacteria samples isolated to correct the microbial contamination in an *in situ* study of protein protection in order to confirm the above indicated relationship as well as to measure the errors in the supply of different nutrients from SAB, when LAB is used as reference and ^{15}N as marker.

Materials and methods

Animals and diets

The study was carried out in three wethers equipped with rumen (inner diameter 80 mm) and duodenum T-cannulaes fed in successive periods with three isoproteic diets with 45% oat (*avena sativa*) hay and 55% concentrate (on fresh matter). The concentrate contained corn grain (30%), barley grain (30%), sunflower meal (15%), spring pea (22%), minerals and vitamins (3%). The only difference among diets was that sunflower meal and spring pea (which provided about 55% of the total crude protein (CP) of the concentrate) were included as untreated (diet UT) or treated with solutions of ortophosphoric acid (diet PT) or malic acid (diet MT) plus heat to protect their proteins. The diets were supplied simultaneously to all wethers in the order above indicated. The protective treatments of these both meals as well as all details on their *in situ* digestive use are shown in Díaz-Royón et al. (2015). In brief, these treatments were carried out spraying 4 N solutions (200 ml/kg) of malic acid (268.2 g/l) or orthophosphoric acid (130.6 g/l) and drying the meals in a forced air oven at 120ºC average the first hour, and then allowing it to dry overnight while it cooled off. Ranges of organic matter (OM), CP, neutral (NDF) and acid (ADF) detergent fibres among diets were (g/kg of dry matter (DM)): 880-885, 177-181, 323-326 and 150-154, respectively. Diets were offered at 45 g/kg of metabolic weight in six equal meals (every 4 h), starting at 0900 hours. Animals were housed in individual pens and handled according to ethical guidelines as published in the Spanish Royal Decree 327/2007 (BOE 2007).

Experimental procedures

After an adaptation period of 10 days to each diet and during a total of 12 days (used for *in situ* studies not reported here to measure the efficiency of protein protection) a solution of $(^{15}NH_4)_2SO_4$ (98 atoms% enriched) was continuously infused into the rumen of the wethers

at a rate of 250 ml/day to provide a daily dose (per animal) of 25 mg ^{15}N to label ruminal bacteria. Then, the rumen was manually emptied just before the 0900 meal and ruminal contents were homogenized before taking 1.2 kg sample (fresh matter) of rumen digesta which was squeezed through a double layer of nylon cloth with pore sizes of 46 μm. Samples of LAB and SAB were isolated as described by Rodriguez et al. (2000) and then lyophilised and analysed for OM, total lipids, polysaccharide-glucose, N and ^{15}N abundance.

Chemical analyses

Samples of SAB and LAB were analysed in accord with the methods of the Association of Official Analytical Chemists (AOAC 2000) for concentrations of DM (procedure 934.01), ash (procedure 967.05) and CP (procedure 968.06). A Leco FP-528 combustion analyzer (Leco Corp., St. Joseph, MI) was used for this last analysis. Lipids in bacterial samples were extracted with a chloroform-methanol mixture (2:1, v/v) as described by Folch et al. (1953). Microbial polysaccharides were determined as glucose by the amyloglucosidase-α-amylase method (procedure 996.11; AOAC 2000). The isotope (^{15}N) abundance was determined by mass spectrometry.

Statistical analysis

Comparisons between LAB and SAB composition were made by ANOVA with a split-plot arrangement of treatments considering the diet (*d*) as the whole-plot treatment (which was tested using the wethers (*w*) × diet interaction as the error term) and the type of bacteria (*b*) and its interaction with diet as sub-plot treatments ($y_{ijk} = \mu + d_i + b_j + w_k + d_i \times b_j + d_i \times w_k + \varepsilon_{ijk}$). Orthogonal contrasts were used to analyse differences among diets. As the animals were adult maintained in a controlled environment, the period effect was assumed to be negligible. Simple linear relationships were performed to relate ^{15}N enrichment of SAB and LAB. The fit of present data to the single equation predicting the ^{15}N enrichment in SAB from that in LAB

shown by González et al., (2012) was determined through the mean prediction error (absolute and relative) as indicated by Montgomery and Peck (1982). Effects of the factors were declared significant at $p < 0.05$. All the statistical analyses were performed using the GLM and REG procedures of SAS software, version 9.0 (SAS Inst. Inc., Cary NC).

Results

Compared with SAB, LAB showed consistently lower contents of OM, total lipids and starch-glucose, but greater CP content and ^{15}N enrichment (Table 1). Diets also affected the contents of CP ($p = 0.041$) and lipids ($p = 0.003$) of bacteria, although an interaction bacteria × diet ($p = 0.003$) was observed for this last fraction (Table 1). Differences for CP were associated with the low values recorded in the diet PT which were lower than those of diet MT ($p = 0.010$). The interaction shown for lipids was associated with the different trend between both bacteria types in untreated and treated diets. Thus, whereas in the UT diet the SAB-lipid content was lower than in treated diets the value in LAB was intermediate between those shown in MT and PT diets, which, on the other hand, showed a great difference between both.

Current data of ^{15}N enrichment in LAB and SAB fitted well (Figure 1) to the previous relationship found between both values in works of this team (González et al. 2012). As a consequence, the inclusion of these 9 pairs of values scarcely affected their fitting parameters. Thus, the slope coefficients were similar and the regression coefficient (R^2) only decreased from 0.994 to 0.985 compared with the cited work. The mean prediction error and the relative mean prediction error associated with the inclusion of the new values were 0.0109 (atoms %) and 13.0%, respectively. Values from diet PT provided the worst fit; thus, the above cited values are reduced to 0.0076 and 8.79%, respectively when data from diet PT is excluded of the regression. The independent term of this equation was not different from 0 ($p = 0.280$). The exclusion of this term in the regression leads to a slope of 0.780 (Figure 1).

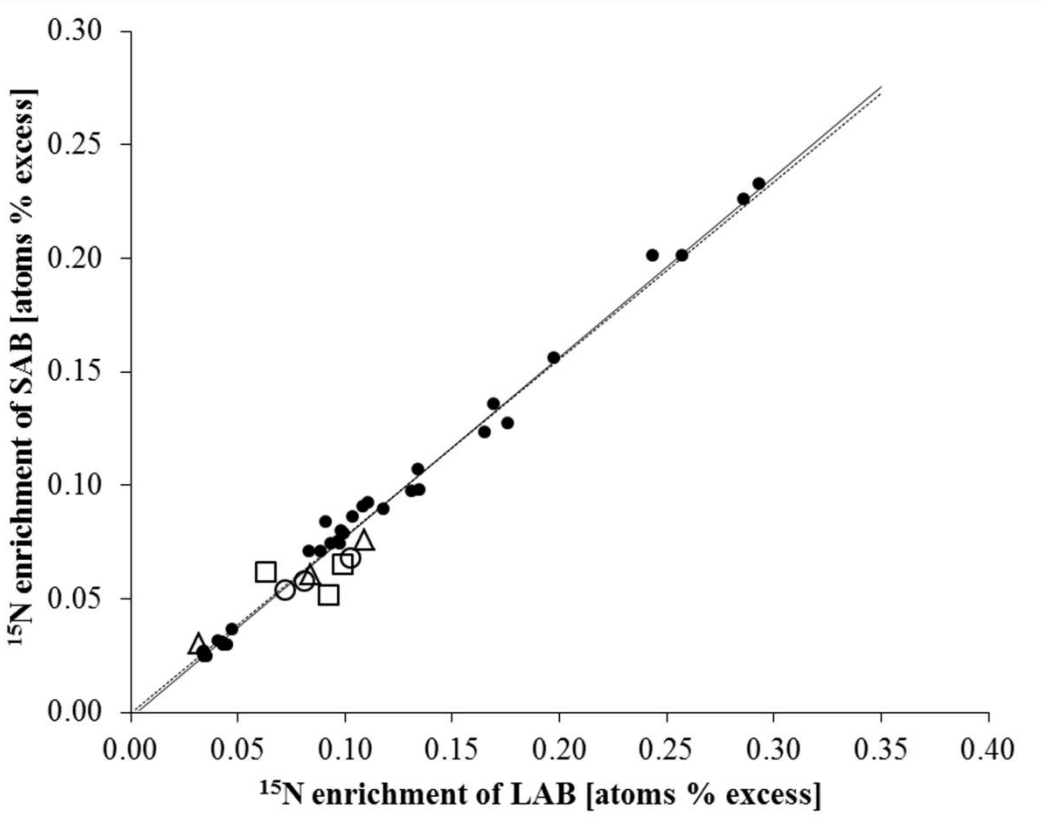

Figure 1. Relationships between the enrichment (^{15}N/ total N; atoms% excess) of solid associated bacteria (SAB) and liquid associated bacteria (LAB). Data from González *et al.* (2012) (●) and from untreated (○), malic acid (Δ), and orthophosphoric acid (□) treated diets of this trial. Equation 1 (——): Y= - 0.0022 (SE =0.0020) + 0.794·X (SE = 0.0156); n = 41; R^2 = 0.985; $p < 0.001$. Equation 2 (- - - - -): Y= 0.780·X (SE = 0.0081); n = 41; $p < 0.001$.

Discussion

Higher concentrations of OM and total lipids in SAB compared to LAB agree with prior results (Merry and McAllan 1983; Legay-Carmier and Bauchart 1989; Rodríguez et al. 2000; González et al. 2012). Also, Vlaeminck et al. (2006) showed that total fatty acid contents were 1.6 to 2.8 times higher in SAB than in LAB. Differences for OM and lipids are evidently associated. Thus, Rodríguez et al. (2000) indicated that differences in lipid concentration

Table 1. Chemical composition (g/kg dry matter) of bacteria isolated from liquid (LAB) and solid (SAB) fractions of rumen contents as affected by diets.

	Diets*						Effects				
	UT		PT		MT		Diet#			Bacteria	
	LAB	SAB	LAB	SAB	LAB	SAB	SEM	C_1	C_2	SEM	*p*
Organic matter	693	777	673	791	713	773	4.7	0.876	0.462	8.3	<0.001
Crude protein	443	406	398	393	448	406	14.1	0.114	0.010	4.8	0.007
Lipids†	111	194	141	245	87.7	223	18.5	0.003	0.013	2.6	<0.001
^{15}N enrichment‡ ($\times 10^{-2}$)	8.54	6.01	8.48	5.98	7.50	5.55	0.341	0.517	0.327	0.379	0.005
Glucose	19.9	23.7	19.6	32.4	20.4	44.1	4.31	0.121	0.228	2.71	0.013

*UT, PT and MT: diets with protein concentrates untreated or treated with ortophosphoric or malic acids plus heat, respectively.

#orthogonal contrasts: C1: UT vs. PT, MT. C2: PT vs. MT.

†significant interaction: SEM = 4.50; $p = 0.003$.

‡atoms %.

contributed to OM differences in 44.7%. A similar value (46.7%) has been shown by González et al. (2012) as the average of four experiments including a total of 24 pairs of bacterial samples. Current results also provided similar values for this contribution in diets UT (49.7%) and PT (46.8%) whereas the MT diet showed a higher contribution (69.3%). Considering current mean diet contents of OM and total lipids of both bacteria types together with those of Rodríguez et al. (2000) and González et al. (2012), performed with identical procedures, the close correlation coefficient recorded (0.741; $n = 26$; $p < 0.001$) show also the previously indicated relation. Merry and McAllan (1983) associated the higher lipid contents in SAB with a higher concentration in lipids or microbial lipid precursors (acetate, fatty acids) in their microenvironment, which can increase lipid synthesis and adsorption of fatty acids onto cells. Rodríguez et al. (2000) associated this higher lipid concentration in SAB with higher proportions of Gram-negative bacteria in this population than in LAB, because bacterial lipids are mainly associated with the phospholipid layer of their cell wall, which is single in Gram-positive and double in Gram-negative bacteria. Consequently, lipid concentration in the cell wall is markedly higher in Gram-negative bacteria (Cummins 1989). Considering that the main effect on bacteria lipid content of the protection treatments was recorded in SAB, the hypothesis of Rodríguez et al. (2000) may contribute to explain their higher contents in diets with treated protein concentrates, as far as protective treatments slow down their degradation increasing the half-life of adherent microcolonies, which in turn may have an effect on their proportion of Gram-negative bacteria.

Differences in the CP contents between SAB and LAB are inconsistent in the literature, existing disagreements among works in the sign of this difference as well as studies showing similar values (González et al. 2012). Reasons for the different CP content of bacteria between PT and MT diets remain unknown. The lower ^{15}N enrichment in SAB compared to LAB agrees with most studies on this subject. Rodríguez et al. (2000) and González et al. (2012) associated

this fact to a lower fixation by SAB of ammonia from the rumen liquid ammonia pool (in which is diluted the infused ^{15}N dose) either by the capture by these microorganisms of N-compounds derived from the local degradation or by diffusion difficulties inside the microbial micro-colonies. The relationships shown in Figure 1 calculated from a total of 7 experiments with different wethers, diets and ^{15}N doses evidence the close correlation between the ^{15}N enrichment of both bacteria types. When the independent term of these equations is restricted to be zero (as it should be when ^{15}N is not infused) the slope of the equation were 0.788 (González et al. 2012) and 0.780 (present values) showing that N fixed by SAB coming from the local degradation may be estimated as 21.2% or 22% of the total fixed N, respectively. González et al. (2012) also showed that the underevaluation error (as %) of the N supply to the animal from the synthesized SAB when LAB are used as reference may be calculated as (1-(^{15}N enrichment in SAB/^{15}N enrichment in LAB)) × 100, and, therefore, these errors are equal to the last cited percentages. Based on the underevaluation for N derived from the current relationship (22%) and the concentration of glucose and lipids in both bacterial populations, mean underevaluations of 43.1% and 59.9% are calculated for the supply from SAB of polysaccharide-glucose and lipids, respectively, if LAB are used as reference. Values for the lipid underevaluation are close to those (59.6%) reported in González et al. (2012).

The equation derived from current and previous data of this team is also supported by that obtained (Figure 2) using mean diet values (n = 41) of ^{15}N enrichment in LAB and SAB obtained in cows (Martin et al. 1994; Yang et al. 2001; Ahvenjärvi et al. 2002; Reynal et al. 2005; Brito et al. 2006, Ipharraguerre et al. 2007), steers (Firkins et al. 1987; Beckers et al. 1995; Kamoun et al. 2014), sheep (Chicunya and Miller 1999, Martín-Urúe et al. 1998, Rodríguez et al. 2000, Ramos et al. 2009; González et al. 2012), and in vitro (Ranilla et al. 2000; Carro and Miller 2002). The independent term of this equation was also not different from 0 (p = 0.162). The exclusion from the regression of the independent term leads to a slope in

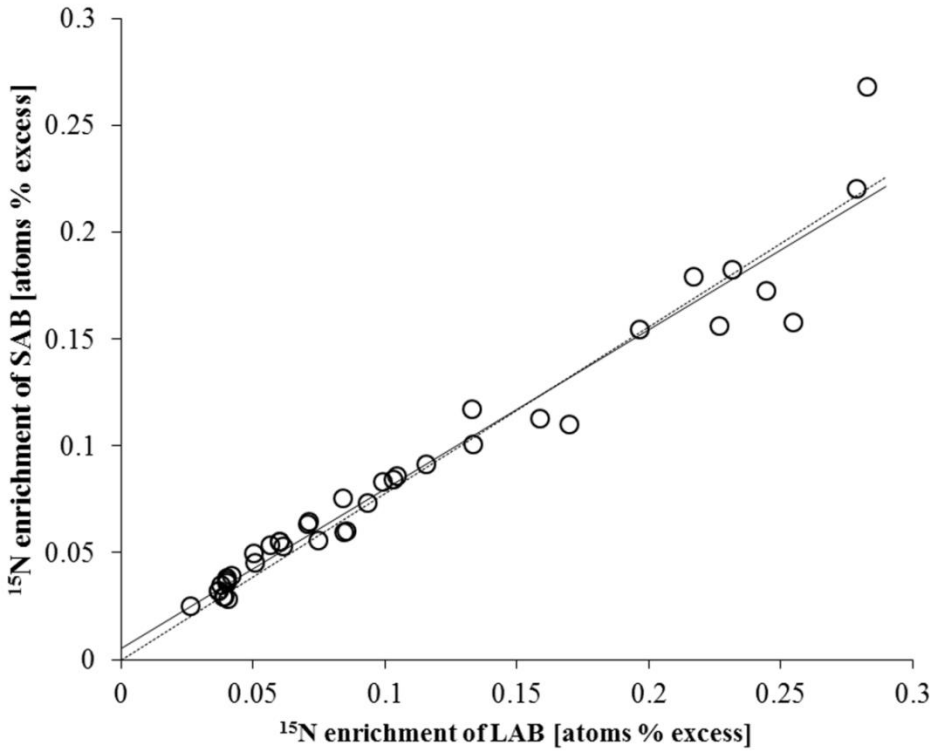

Figure 2. Relationships between the enrichment (^{15}N/ total N; atoms% excess) of solid associated bacteria (SAB) and liquid associated bacteria (LAB) of mean diet values from the literature. Equation 1 (——): Y= 0.0051 (SE = 0.0034) + 0.749·X (SE = 0.0267); n = 41; R^2= 0.984; $p < 0.001$. Equation 2 (- - - - -): Y= 0.779·X (SE = 0.0148); n = 41; $p < 0.001$.

the regression and, therefore, to a fixed relation between the ^{15}N/ total N ratio in SAB and LAB of 0.779 (Figure 2). As a consequence, the proportion of N fixed by SAB coming from the local particle degradation as well as the underevaluation error of the N supply from SAB when LAB are used as reference (22.1% for both parameters) are practically identical to those derived from the current work, in spite of the additional variation sources incorporated (animal type, bacteria isolation methods, ^{15}N enrichment of ammonia sources, teams, etc.). The mean error indicated above, together with a good knowledge of the proportion of both bacteria populations in the post-ruminal flow, may be used to obtain more accurate estimates of the microbial protein synthesis in the rumen. Unfortunately, information about the contribution of SAB and LAB (or

other microorganisms) to the microbial flow from the rumen is still scarce and needs more attention. The differences between LAB and SAB in chemical composition and specially the lower ^{15}N/total N ratios in SAB than in LAB determine large underevaluations in the estimates of the nutrient supply to the ruminant from SAB when only LAB isolates are used as reference samples. Data from this trial and from literature support a previous prediction of the CP underevaluation of about 22%. These underevaluations will be larger for the supply of glucose (43.1%) and lipids (59.9%) as a consequence of the higher concentrations of both nutrients in SAB compared to LAB. These observations should be considered to improve the accuracy of the microbial nutrient supply estimates and in the protein evaluation for ruminants.

Acknowledgments

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CAPITULO 5

EFFECTS OF PROTEIN PROTECTION WITH ORTHOPHOSPHORIC OR MALIC ACIDS AND HEAT ON FATTENING LAMB DIETS

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En revisión en Small Ruminant Reseach

Abstract

The objective of this research was to examine the effects of treating sunflower and spring pea meals with orthophosphoric or malic acid solutions and heat on growth performance, concentrate intake, and carcass yield and fatness of growing-fattening lambs. Ninety "Entrefino" cross male lambs from three commercial farms (average initial body weights (BW) = 14.6, 15.3 and 13.3 kg) were randomly assigned to five diets with different acid treatment and protein levels, and fattened to an average slaughter weight of 25 kg. Protein sources in the control concentrate (C; crude protein (CP) = 18%) were soybean meal and untreated sunflower and spring pea meals. In three of the experimental concentrates, orthophosphoric acid-treated meals substituted untreated sunflower and spring pea meals (Orthophosphoric Control, PC; CP = 18% dry matter basis (DM)), and soybean meal was partially (Medium Substitution Orthophosphoric, MSP; CP = 16.7%) or totally removed (Total Substitution Orthophosphoric, TSP; CP = 15.6%). In addition, in one concentrate orthophosphoric acid was replaced by malic acid to protect these meals (Medium Substitution Malic, MSM; CP = 16.7%). Wheat straw (roughage source) and concentrate were offered *ad libitum*. No effect associated with the CP level was observed on any parameter. This suggests that with protected proteins it is possible to feed concentrates with 15.6% CP (on DM) reducing the need to include vegetable protein meals as well as the quality of the protein concentrates. Lambs feed MSM had higher average daily gains (15.2%; P = 0.042), and better hot carcass yields (1.3 percentage points; P = 0.037) than lambs feed MSP. This probably can be explained by ruminal malate actions and by greater protection effects obtained with malic acid.

Keywords: Protein protection; Sunflower meal; Spring pea; Malic and orthophosphoric acids; Heat

Introduction

Sunflower meal is a co-product of sunflower oil extraction; with variable content of crude protein (CP) (31-37% as DM) depending upon the extent of de-hulling of the seed (Sauvant et al., 2004). Its protein is highly degradable in the rumen, normally above 80% (Arroyo et al., 2012; Gonzalez et al., 1999; Woods et al., 2003). Since sunflower meal protein is characterized by a relative lysine deficiency and a high content in sulphur amino acids and tryptophan, it complements well the aminoacid profile of leguminous seeds (FEDNA, 2010). Due to their high protein content, the European Union has promoted the production of peas (*Pisum sativum*; Aufrere et al., 2001) although its proteins have the limitation to be also highly degradable in the rumen (INRA, 2007; Khorasani et al., 2001; Petit et al., 1997). Therefore, to reduce this degradability without reducing the intestinal digestibility of the by-pass protein is of interest to improve the use of these both meals. In this sense, Díaz-Royón et al. (2015) reduced CP effective degradability values (corrected by the ruminal microbial contamination) of sunflower meal from 80.1% to 69.1% and to 59.4% treating with orthophosphoric acid or malic acid plus heat, respectively. Similarly, the reductions for spring pea were from 88.1% to 75.3% and to 73.3%, respectively.

The objective of this research was to examine the effects of treating sunflower and spring pea meals with orthophosphoric or malic acid solutions and heat on growth performance, concentrate intake, and carcass yield and fatness of growing-fattening lambs.

Materials and methods

Animals, treatments, and experimental procedures

The experiment was carried out at "Los Campos de Prácticas of the E.T.S.I. Agrónomos". Animals' management was approved by the Animal Ethics Committee of Universidad Politécnica de Madrid and was carried out in accordance with the Spanish

guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes). Ninety weaned "Entrefino" cross male lambs were used from three commercial farms (30 lambs each). Average initial body weight (BW) was 14.62 ± 3.21; 15.3 ± 2.31, and 13.3 ± 1.89 kg (mean ± standard deviation (SD)) for farm A, B and C, respectively. Lambs blocked by farm and initial BW were randomly distributed to pens of three lambs each in a factorial design of five dietary treatments with two replicates (pens) of each treatment per farm. The three lambs of each group were housed in slatted floor pens (1.5 m^2) equipped with two feeders (one for concentrate, another for straw), and an automatic drinker. Before weaning, lambs were vaccinated against enterotoxaemia and dewormed with Ivermectin. At the beginning of the experiment lambs were dewormed again with Closantel. Commercial spring pea and semi-decorticated sunflower meals were treated with solutions (200 ml/kg) of orthophosphoric acid (1.33 M; 130.6 g/l) or malic acid (2 M; 268.2 g/l) and heat (120ºC) following the procedure indicated in Díaz-Royón et al. (2015).

The five experimental diets consisted of wheat straw (roughage source) and one of five concentrate supplements. Concentrates were formulated to be isoenergetic and were designed to meet INRA (2007) nutrient recommendation for male growing-fattening lambs with moderate growth potential. Concentrate ingredients and chemical composition are presented in Table 1. Contents (g/kg of dry matter (DM)) of CP, neutral detergent fibre (NDF) and acid detergent fibre (ADF) in wheat straw were 36.0, 791 and 508, respectively. Protein sources in the control concentrate (C; CP = 18%) were soybean meal, and untreated sunflower and spring pea meals. In three of the experimental concentrates, orthophosphoric acid-treated meals substituted untreated sunflower and spring pea meals (Orthophosphoric Control, PC; CP = 18%), and soybean meal was partially (Medium Substitution Orthophosphoric MSP; CP = 16.7%) or totally removed (Total Substitution Orthophosphoric, TSP; CP = 15.6%). In

addition, in one concentrate orthophosphoric acid was replaced by malic acid to protect (Medium Substitution Malic, MSM; CP = 16.7%). The chemical composition and protein fractions of both untreated and treated sunflower and spring pea meals has been shown in Díaz-Royón et al. (2015). Both sunflower and pea meals supplied 31.1, 30.9, 33.0, 35.8 and 33.1% of the total CP in C, CP, MSP, TSP and MSM, respectively.

Table 1. Ingredient and chemical composition (% unless otherwise noted) of the experimental concentrates

Item	C[a]	CP	MSP	TSP	MSM
Ingredient composition (%)					
Ground barley	53.2	53.2	55.9	58.5	55.2
Ground corn	13.1	13.1	13.8	14.6	13.7
Sunflower meal	10	0	0	0	0
Sunflower orthophosphoric meal	0	10	10	10	0
Sunflower malic meal	0	0	0	0	10.4
Spring pea	10	0	0	0	0
Spring pea orthophosphoric	0	10	10	10	0
Spring pea malic	0	0	0	0	10.4
Soybean meal	6.80	6.80	3.40	0.00	3.40
Lard	3.00	3.00	3.00	3.00	3.00
Calcium carbonate	2.50	2.50	2.50	2.50	2.50
Ammonium chloride	0.50	0.50	0.50	0.50	0.50
Salt	0.30	0.30	0.30	0.30	0.30
Sodium sulfate	0.35	0.35	0.35	0.35	0.35
Vitamin-Mineral Premix	0.30	0.30	0.30	0.30	0.30
Chemical composition (% of DM basis)					
Dry matter	89.6	89.7	89.2	89.8	91
Organic matter	94.3	93.7	94.1	94.1	93.8
Crude protein	18.1	18.1	16.9	15.6	16.7
NDF	21.7	18.4	18.5	19.5	18.4
ADF	7.24	7.04	8.33	7.94	8.08
Lignin (sa)	1.35	1.47	1.95	2.06	1.82
Eter extract	5.51	5.5	5.52	5.54	5.53
UFV[b](/kg DM)	1.11	1.1	1.12	1.11	1.10

[a]C = control concentrate; CP = control orthophosphoric concentrate; MSP = medium substitution orthophosphoric; TSP = total substitution orthophosphoric; MSM = medium substitution malic concentrate.

[b]Unites Fourrageres Viande calculated according to values from Tables INRA (2007)

feeders daily and twice weekly, respectively. Lambs were individually weighed every two weeks throughout the experiment right before feed was offered. Concentrates orts by lamb pen were removed and weighed every two weeks. The average daily intake of concentrate by lamb pen was calculated as the difference between the total amounts of concentrate offered and refused. Individual average daily gain (ADG) was calculated using the initial and final measurements of BW, and average feed conversion ratio (FCR) was calculated as kg of concentrate intake (DM basis)/kg body gain.

The length of the experiment was variable depending on the lamb initial BW. Lambs with higher initial BW were slaughtered at a commercial plant at day 37, whereas lambs with lower initial BW were slaughtered at day 58. Animals fasted during 2 h, stunned with electricity, and then slaughtered. Average final BW were 25.2 ± 3.32, 25.2 ± 3.45, and 25.61 ± 2.79 for farm A, B and C, respectively. The commercial carcass, with kidneys and perirenal fat was weighed while still warm to register hot carcass weight, and then kept at 4°C during 24 h. Carcass was then weighed again to record cold carcass weight. Dorsal-fat (DF; European Community, 1994) and kidney-pelvic-fat (KPF; Colomer-Rocher et al., 1988) were also evaluated. Finally, the hot (HCY) and cold carcass yield (CCY) was calculated as a percentage of the final BW.

Analytical methods

Feed samples were analysed by triplicate according to the AOAC (2000) as follows: DM (AOAC 934.01), ash (AOAC 967.05), and ether extract (AOAC 920.39). CP was analysed using a Leco FP-528 combustion analyzer (Leco Corp., St. Joseph, MI, USA) and estimated by 6.25 x Dumas N (AOAC 2000, method 968.06). Fibre was analysed sequentially with the Ankom system (Model 220, Ankom Technology Corp., Macedon, NY, USA) as follows: NDF (Van Soest et al., 1991), ADF and acid detergent lignin (Robertson and Van Soest, 1981). NDF

and ADF were expressed inclusive of residual ash and NDF analyses were performed with a heat stable alpha-amylase and without sodium sulphite.

Statistical analysis

Data were analyzed using a factorial design considering initial BW as covariate and farm of origin as block. Data on concentrate intake and FCR were analyzed using pen as experimental unit, while ADG, carcass yield, DF, and KPF data were analyzed with lamb as experimental unit. The model included initial BW, farm of origin, treatment, and interactions initial BW × farm of origin, initial BW × treatment, and farm of origin × treatment as fixed effects. For data estimated using individual measurement, the interaction initial BW × farm of origin × treatment was included on the models. Furthermore, treatments were compared through the following contrasts: C *vs.* PC, MSP and TSP; PC *vs.* MSP and TSP; MSP *vs.*TSP; C *vs.* MSM; MSP *vs.* MSM. All the statistical analyses were done using the GLM procedure of SAS for Windows, version 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $P < 0.05$ while those with $0.05 < P < 0.1$ were considered trends.

Results

Results of concentrate intake and lamb performances are summarized in Table 2. Concentrate intake, ADG, FCR, carcass yield, and carcass fatness (DF and KPF) of lambs were similar among treatments. However, lambs fed MSP concentrate grew 15.2% less (34 g/d) than lambs fed MSM ($P = 0.042$). Intakes were affected by farm of origin ($P = 0.010$) and initial BW ($P = 0.043$). Concentrate intakes were 786, 813, and 783 g/d for lambs from farms A, B, and C, respectively. Moreover, an interaction between initial BW and farm of origin was observed ($P < 0.012$). Lambs with greater initial BW consumed more concentrate in farms A and B; whereas lambs in farm C showed lower concentrate intakes. Average daily gain was also affected by farm of origin ($P <0.001$; 231 *vs.* 203 *vs.* 268 g/d for farm A, B, and C,

Table 2 Effect of treatments on concentrate intake, average daily gain (ADG), feed conversion ratio (FCR), carcass yields, and carcass fatness

	Concentrates						P value main effects			*P* value of contrasts[e]				
Item[a]	C	PC	MSP	TSP	MSM	SED	IBW	Farm	Diet	1	2	3	4	5
Intake[b](g/d)	795	792	751	741	815	36.3	0.043	0.010	0.265	0.279	0.168	0.796	0.595	0.103
ADG (g/d)	238	233	224	216	258	16.4	<0.001	<0.001	0.119	0.312	0.395	0.617	0.221	0.042
FCR[c]	3.42	3.42	3.51	3.47	3.14	0.258	0.874	0.121	0.542	0.823	0.715	0.862	0.241	0.132
HCY (%)	46.5	46.2	46.3	46.7	47.6	0.63	0.07	0.23	0.176	0.913	0.604	0.477	0.071	0.037
CCY (%)	45.3	44.9	45.0	45.5	46.3	0.65	0.116	0.296	0.253	0.803	0.542	0.439	0.133	0.057
DF[d]	2.22	2.13	2.13	2.13	2.25	0.138	<0.001	0.036	0.844	0.441	0.986	0.944	0.818	0.368
KPF	2.39	2.22	2.16	2.33	2.47	0.209	0.154	0.080	0.601	0.386	0.897	0.423	0.700	0.147

[a]C = control concentrate; CP = control orthophosphoric concentrate; MSP = medium substitution orthophosphoric; TSP = total substitution orthophosphoric; MSM = medium substitution malic concentrate; SED = Standard error of the difference; IBW = Initial body weight; HCY= hot carcass yield; CCY = cold carcass yield; DF = dorsal fat (ranged from 1 to 5; European Community, 1994); KPF = kidney pelvic fat (ranged from 1 to 5; Colomer-Rocher et al., 1988).
[b]Interaction IBW x Farm ($P = 0.012$).
[c]FCR = kg concentrate intake /kg body gain. Interaction IBW x Farm ($P = 0.043$).
[d]Interaction IBW x Farm ($P = 0.039$).
[e]Contrasts: 1: C *vs.* PC, MSP, and TSP; 2: PC *vs.*MSP, and TSP; 3: MSP *vs.*TSP; 4: C *vs.* MSM; 5: MSP *vs.* MSM.

respectively) and by initial BW in such a way that ADG increased 7.5 g per kg of additional initial BW ($P < 0.001$). Although FCR was unaffected by experimental concentrate, initial BW, or farm of origin, lambs with lower initial BW showed greater FCR, except lambs from farm A (P value interaction initial BW × farm of origin = 0.012). Carcass yields (hot and cold) were unaffected by concentrate, although hot carcass using the MSM concentrate yielded 1.3 percentage points more than did with the MSP ($P = 0.037$). Dorsal-fat did not differ among concentrates, but it was affected by initial BW and farm of origin ($P < 0.05$). An interaction between initial BW and farm of origin was also detected ($P = 0.039$).

Discussion

Concentrate intake, ADG, and FCR values in this study are comparable to those of other Spanish studies when lambs were raised under intensive systems and offered concentrates and cereal straw *ad libitum*, and slaughtered between 25-27 kg (Flores, 2004, Manso et al., 2006, Mungoi et al., 2012). The effect of farm of origin on ADG indicates differences in growth potential among animals from different farms. Lambs from farm C presented 32 and 16% greater ADG than lambs from farm B and A, respectively. The lack of interactions farm × diet and initial BW × diet suggests that in commercial feedlots where lambs are fattened until they reach the slaughter weight as demanded by the market (25 kg) concentrates need not to be adjusted neither for growth potential nor initial BW.

The lack of increases in performance values between the PC concentrate and the C concentrate could indicate that quantity and profile of amino acids absorbed by lambs fed the C concentrate with 18.1% CP was sufficient to meet requirements of "Entrefino" cross male lambs. This CP level will be equivalent to 16.7% in these diets assuming a straw consumption about 10% of the total intake (INRA, 1978). The current work supports previous finding in which supplemental RUP was apparently not required by early weaned lambs (Beauchemin et al., 1995; Glimp et al., 1967; Tufarelli et al., 2009).

On the other hand, the similar values of growth performance among concentrates in spite of the progressive reduction from the controls (C, CP) of their CP content (6.7% in MSP and MSM and 13.6% in TSP) indicates that inclusion of treated meals could allow a decrease in the CP concentration of concentrate until 15.6% (equivalent to 14.4% in the diet) without affecting concentrate intake, ADG, and FCR. These results may be associated with a higher efficiency of the ruminal microbial synthesis with the reduction of the proportion of CP degraded in the ruminally fermented OM (NRC, 2001). This reduction will be a consequence of both the decrease in the CP supply by the SBM reduction and the decrease of the CP degradability of the treated meals (Díaz-Royón et al., 2015). Thus, this last study showed that the RUP contents of the used meals increased in 55.3% and 104% (sunflower meal) and 108% and 124% (spring pea), when they were treated with orthophosphoric acid or malic acid plus heat, respectively. Present results concur with data published by Glimp et al. (1967) who heated soybean meal and improved gains of lambs fed a 12% CP diet to a point where they were comparable to those obtained on a 17% CP diet.

The higher values observed in concentrate MSM compared to MSP for ADG (258 vs. 224 g/d) may be associated with a more intense effect on the ruminal microbial synthesis due to the reduction of CP degradation (NRC, 2001). It is also possible a superior supply of digestible amino acids in the small intestine on the MSM diet. As previously indicated, Díaz-Royón et al. (2015) showed a higher effectiveness when sunflower meal or spring pea meal were treated with malic acid compared to orthophosphoric acid. Arroyo et al. (2013) also reported a greater effectiveness treating sunflower meal with malic acid than with orthophosphoric acid because malic acid treatment led to a higher intestinal digestibility. In addition, positive effects of malic acid supply on ruminal fermentation should be considered. Malic acid is an intermediate of the randomizing pathway of propionate production in the rumen, and its supply has been shown to increase propionate concentrations both in vitro (Carro

and Ranilla, 2003; Tejido et al., 2005) and *in vivo* (Kung et al., 1982; Khampa et al., 2006). An increase in propionate production would result in increased energy efficiency and therefore in improved ruminant performance. Malic acid can also stimulate the lactate uptake by *Selenomonas ruminantium*, thus reducing or preventing the risk of drop of ruminal pH (Castillo et al., 2004). In several studies malic acid supplementation to meat ruminants has been shown to have a favorable impact on ADG. Martin et al. (1999) reported that supplementing malic acid at rates of 3.7 and 7.1 g/kg diet DM to feedlot steers significantly improved ADG and feed to gain ratio, and Flores et al. (2003) found similar results in lambs supplemented with malate salts at 2 and 4 g/kg diet DM. The concentration of malic acid in the present study was 12.0 g/kg diet DM, which is higher than those used in the above cited studies. Therefore, it could be speculated that the greater ADG observed in MSM-fed lambs may be associated with a positive effect of malic acid on ruminal fermentation.

Hot and cold carcass yields as well as dorsal-fat and kidney-pelvic-fat did not differ between concentrates, which concurs with results of Beauchemin et al. (1995). These authors reported no effects of CP content or protein degradability of the diets on dressing percentage and kidney fat of lamb carcasses. The greater fed efficiency indicated in MSM concentrate compared with MSP is also supported by the greater carcass yields observed in this diet.

Conclusion

Results of this experiment demonstrated that treated spring pea and semi-decorticated sunflower meals could completely replace soybean meal in concentrates of growing-fattening lambs with moderate growth potential and that the CP percent in the concentrate could be decreased to 15.6% using treated meals without affecting intake and lamb performance.

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CAPITULO 6

DISCUSIÓN GENERAL

6.1. Eficacia del tratamiento combinado de las harinas de girasol y guisante con soluciones ácidas y calor.

La elección de las harinas de girasol y de guisante como alimentos a tratar en este estudio fue debida a la popularidad de ambos cultivos en la agricultura española, siendo pues la principal oleaginosa y la principal leguminosa grano que se producen en España, respectivamente. Como ya se señaló, las proteínas de ambos alimentos presentan la limitación de una elevada degradabilidad ruminal. Además, debido a sus diferencias en el contenido de aminoácidos esenciales, estas harinas se complementan muy bien cuando se utilizan conjuntamente.

Previamente, este grupo de investigación (Arroyo y col., 2013) incrementó en un 267% el contenido de PNDR de una harina de girasol tratada sucesivamente con soluciones ácidas (ácidos ortofosfórico o málico; 400 ml/kg) y calor. Sin embargo, la extensa duración e intensidad del tratamiento térmico aplicado (6 horas a 150 ºC) ocasionan que el coste asociado con este, sin incluir el coste de los químicos, ascendiese a 30 euros/tm (Arroyo, 2012). Evidentemente este coste sería inferior en condiciones industriales de producción frente a las condiciones de laboratorio (secado en estufa) en que se realizó. No obstante, a fin de rebajar este coste, el objetivo de este estudio fue determinar los efectos de este tratamiento combinado en la degradabilidad de harinas de girasol y guisante, pero disminuyendo el tratamiento térmico (120 ºC durante 1 hora) en base a reducir la cantidad de solución ácida (200 ml/kg) pero sin variar la dosis de ácidos a aplicar duplicando la concentración de la solución. El volumen de solución utilizado por Arroyo y col. (2013) fue determinado a partir de un estudio previo llevado a cabo es este laboratorio (Arroyo y col., 2011) en el que se evaluó el efecto de la aplicación de 5 volúmenes de solución de ácido ortofosfórico en la degradabilidad in vitro de la PB de la harina de girasol (40,2, 41,9, 43,8, 44,2 y 43,4% de PB degradada con volúmenes de solución aplicados de 80, 160, 240, 320, y 400 ml/kg de alimento, respectivamente; P =

0,063), no encontrando estos autores ninguna ventaja adicional al aumentar el volumen de solución por encima de los 240 ml/kg de harina.

El tratamiento combinado con ácidos y calor aumentó la fracción de PNDR en ambas harinas en este estudio; sin embargo, en la harina de girasol este incremento fue sensiblemente inferior al obtenido por Arroyo y col. (2013). Por otra parte, los tratamientos no afectaron la digestibilidad intestinal de la proteína de las harinas testadas, al contrario de lo observado por estos autores. Como resultado, si bien la fracción de proteína dietaría absorbida en el intestino aumentó significativamente, lo hizo básicamente como consecuencia de la reducción de la degradabilidad ruminal. De forma concordante con Arroyo y col. (2013), el ácido málico mostró mejores resultados que el ácido ortofosfórico en la protección de la harina de girasol. Igualmente, aunque no hubo diferencias estadísticas entre ácidos, el uso de ácido málico dio lugar a un incremento en la eficiencia de protección en la harina de guisante un 15.6% superior al correspondiente al ácido ortofosfórico una vez corregida la contaminación microbiana. Tras esta corrección, las fracciones de PNDR y proteína dietaría absorbida en el intestino en la harina de girasol incrementaron un 104,0 y un 113,8 % con el ácido málico y un 55,3 y un 59,9% con el ácido ortofosfórico, respectivamente. En la harina de guisante, el tratamiento de protección incrementó en 116,0 y 149,2% la PNDR y la proteína dietaría absorbida en el intestino, respectivamente, independientemente del ácido utilizado. La eficiencia de protección en la harina de girasol medida como proteína dietaría absorbida en el intestino fue igualmente inferior a la obtenida por Arroyo y col. (2013) que observaron un incremento de 4,3 veces, mientras que en el presente estudio este aumento fue de 2,1 y 1,6 veces, dependiendo del ácido utilizado (ácidos málico y ortofosfórico, respectivamente). Estos resultados pueden ser debidos a los menores niveles de humedad y calor aplicados en este experimento.

Los tratamientos de protección también reducen la fermentación de la MO en el rumen lo que evidentemente debe tener consecuencias sobre la síntesis de PM en el rumen. Aplicando

el sistema PDI (INRA, 2007), el valor proteico de la proteína aumentó en un 45,5 y un 20,1% en la harina de girasol, y en un 4,95 y en un 1,79% en la harina de guisante, al proteger estas con ácido málico u ortofosfórico, respectivamente. La baja eficiencia en la protección de la harina de guisante está en parte relacionada con la baja digestibilidad intestinal de la proteína observada en esta harina sin tratar o tratada. Sorprendentemente, los valores de digestibilidad de la proteína obtenidos en este experimento son 2,5 veces inferiores a los valores tabulares publicados para la harina de guisante (91%, Sauvant y col., 2004). Esta baja digestibilidad puede ser atribuida a la presencia de un factor antitripsina no termolábil en esta partida de harina de guisante. No obstante este hecho, los resultados a nivel ruminal de este trabajo tienden a indicar que estos tratamientos son principalmente de aplicación en alimentos con alto contenido en proteínas ya que el desplazamiento del sitio de digestión del rumen al intestino con el tratamiento es superior para la proteína que para las restantes fracciones del alimento. Además, en este tipo de alimentos se reduce en mayor medida el ratio proteína fermentada/MO fermentada en el rumen lo que se asocia con una mayor eficiencia de crecimiento microbiano en el rumen (NRC, 2001) y proporcionalmente se reducen los efectos sobre otras fracciones fermentables distintas de la proteína al ser la cuantía de estas más limitada.

El estudio de electroforesis permitió algunas observaciones de cierto interés, especialmente considerando el carácter no cuantitativo de esta prueba. Así para ambos alimentos puede apreciase una degradación casi total de las albuminas, así como la ausencia de protección de las mismas con los tratamientos. Los cambios observados en las muestras compuestas representativas de la fracción by-pass respecto al total de proteínas insolubles (muestras 0 h) también evidencian la degradación casi total de algunas proteínas (más intensa en el guisante) y la degradación parcial de otras. Estos cambios deben implicar alteraciones en el perfil de AAs que abandonan el rumen como indican Arroyo y col. (2013). Dentro de estos cambios, en el guisante destaca una mayor degradación de vicilinas y convicilinas que de

leguminas, acorde con la presencia en estas últimas de puentes disulfuro, ausentes en las primeras (Casey y Domoney, 1999). Finalmente, la ausencia de bandas polipectidicas en las muestra indigeridas en el intestino tiende a indicar que la PB no digerida observada *in situ* debe corresponder principalmente a compuestos de N no proteico (probablemente asociados a la fibra) o a péptidos demasiado pequeños no detectables con esta técnica. La presencia en guisante de bandas difusas escasamente resueltas en áreas de bajo peso molecular parecen así mismo indicar la presencia de péptidos no digeridos lo que sería concordante con la presencia de un factor antitripsico no termolábil (probablemente taninos) en esta harina de guisante.

La prueba de cebo de corderos no resultó claramente determinante de la mejora del valor nitrogenado de las harinas testadas con los tratamientos de protección. Así, los similares rendimientos productivos observados entre el concentrado control y el control ortofosfórico muestran una falta de respuesta a un suplemento adicional de aminoácidos digestibles. La incapacidad de mejorar las tasas de crecimiento de las dietas con proteínas protegidas puede ser debida a la importante contribución de la proteína microbiana formada a partir de la gran cantidad de materia orgánica fermentada en el rumen (Ludden y col.1995). En un estudio con terneros canulados, Ludden y Cecava (1995) mostraron que al alimentar con fuentes de proteínas resistentes a la degradación ruminal no aumentaba el flujo de aminoácidos metabolizables al intestino delgado. Wessels y Titgemeyer (1997) indicaron que cuando aumenta el contenido en N by-pass de las fuentes proteicas, el flujo de proteína microbiana disminuye, contrarrestando el mayor flujo de N dietario.

La falta de respuesta al aumentar al contenido en proteína by-pass de las dietas también pudo ser debida a que la concentración proteica de la dieta control fuera excesiva en relación con las posibilidades de crecimiento de los corderos utilizados en este ensayo, si bien los piensos se diseñaron conforme a las recomendaciones del INRA (1988). Sin embargo, los valores similares de crecimiento, consumo e índice de conversión (IC) de los concentrados

tratados con ácido ortofosfórico frente al concentrado testigo si demuestran la capacidad de sustitución de la harina de soja por las harinas protegidas con ácido ortofosfórico, así como la posibilidad de reducir simultáneamente por este método la concentración de PB en el pienso hasta un nivel de 15,6 % sobre MS, ambas cosas a pesar de la baja digestibilidad intestinal observada para el guisante de primavera. Los resultados obtenidos muestran que los requerimientos de corderos de crecimiento moderado quedan cubiertos con este nivel de PB en los piensos cuando el aporte de aminoácidos digestibles es el adecuado. Si consideramos que el consumo de paja representa aproximadamente un 10 % de la MS total ingerida (INRA, 1988), el contenido en proteína de la ración total correspondería a un 14,5 % sobre MS. Este nivel de concentración proteica coincide con las recomendaciones del NRC (1985), que propone niveles de 14,5 % de PB para maximizar el crecimiento de corderos destetados precozmente. Por el contrario, este nivel se aleja del recomendado por el INRA (1988), que indica que las dietas no deben contener menos de 18 % de PB sobre MS del destete hasta los 25 kg de peso.

Resultados semejantes también se han descrito en ganado vacuno en crecimiento. Un aumento del nivel de proteína en dietas para terneros no siempre llevó consigo un incremento en la ganancia media diaria (GMD). Así, en nueve trabajos descritos por Ferret y col. (2006), se observó una respuesta positiva en el crecimiento en cuatro de ellos, mientras que en el resto no se observó mejoría alguna. Devant y col. (2000) comprobaron que reducir del 17 al 14% sobre MS el nivel de PB en pienso en terneras cruzadas (101 kg peso inicial) no modificaba la GMD ni el consumo, mientras que la excreción de N se redujo a la mitad. En terneros Holstein en crecimiento (de 151 a 277 kg) alimentados con una dieta con 90% de concentrado, Lana y col. (1997) no observaron mejora alguna en GMD, pero hubo una disminución en el consumo y una tendencia a aumentar el IC cuando el nivel de PB de la dieta disminuyó de 16,6 a 13,5 % sobre MS.

La superior eficacia de la dieta con ácido málico frente al concentrado tratado con ácido ortofosfórico con el mismo nivel de PB podría atribuirse a la mayor eficacia de protección del ácido málico que fue discutida anteriormente. No obstante, dada la falta de respuesta al aumentar el aporte de aminoácidos digestibles, indicada al comparar la dieta control frente a la dieta control ortofosfórico, parece más probable que la razón por la que el concentrado que contiene ácido málico haya producido corderos con mejores velocidades de crecimiento y rendimientos a la canal que el concentrado con ácido ortofosfórico, sea atribuible a los efectos beneficiosos de la incorporación de ácido málico en la fermentación ruminal.

El ácido málico es un producto intermedio de una de las vías metabólicas (succínica) por la cual el piruvato se transforma en ácido propiónico. La incorporación de malato a la dieta reduciría la importancia cuantitativa de la vía que formaría lactato (Caja y col., 2003; Ranilla y Carro, 2003) y que se asocia con una reducción de la eficacia en la síntesis de PM. Además, Nisbet y Martin (1990, 1993) observaron *in vitro* que la adición de ácido málico estimula el crecimiento de la bacteria ruminal *Selenomonas ruminantium* incrementando la utilización del ácido láctico en el rumen, lo que ayuda a prevenir los problemas de acidosis en dietas muy concentradas. La administración de malato produce un aumento significativo de la producción de butírico y propiónico, mientras que la relación acético: propiónico disminuye linealmente con niveles crecientes de malato (Carro y Ranilla, 2003). Dada la pobre utilización del acetato en el cebo de rumiantes, la disminución de esta relación tiene claros efectos positivos sobre el rendimiento del animal y, a través de éste, sobre el consumo y el IC.

6.2. Mejora de la estimación del aporte de nutrientes microbianos al intestino.

La necesidad de corregir la contaminación microbiana en los estudios *in situ* conlleva el aislamiento y determinación de la composición química de las bacterias, de forma que son numerosos los datos obtenidos sobre esta composición por éste u otros equipos de investigación. Los resultados obtenidos en este experimento validan la ecuación de regresión

entre los enriquecimientos en ^{15}N de las bacterias adherentes y libres publicada previamente por este equipo (Gonzalez y col, 2012). Esta ecuación permite predecir la subestimación de los aportes de proteína microbiana correspondientes al flujo postruminal de bacterias adherentes (mayoritarias en el rumen) al utilizar ^{15}N como marcador y, como ha venido siendo habitual, las bacterias libres como referencia. Este trabajo muestra que el aporte de PB microbiana a partir de las bacterias adherentes es subestimado en un 22% cuando se usan las bacterias libres como referencia. Ya que la proteína microbiana representa la mayor parte de la proteína que sale del rumen (Clark, 1992), una mejora en la estimación de ésta podría suponer un avance en la alimentación practica de rumiantes. La subvaloración indicada no se limita a los aportes de proteína microbiana sino también a otros nutrientes cuya concentración es superior en las bacterias adherentes que en las libres y que han sido objeto hasta el momento de una escasa atención por los sistemas de alimentación de rumiantes. Así, esta subvaloración alcanzaría el 43,1% para la glucosa de los polisacáridos, siendo ésta a nuestro conocimiento la primera indicación a este respecto, y el 59,9% para los lípidos. Este último valor es prácticamente repetitivo con el valor medio (59,6%) correspondiente a los múltiples estudios indicados en González y col. (2012).

Dadas las numerosas condiciones de alimentación y los múltiples ovinos utilizados en los experimentos en los que se han determinado los enriquecimientos en ^{15}N de ambas poblaciones de bacterias, la regresión obtenida debe considerarse resultado de una ley general derivada del funcionamiento de las microcolonias a nivel de la captación de compuestos nitrogenados degradables provenientes de la degradación local (NH_3 o AA) y de la difusión del NH_3 del líquido ruminal hasta sus microorganismos integrantes. Este carácter general se confirma por la relación similar obtenida a partir de valores bibliográficos.

La ecuación de regresión obtenida entre los enriquecimientos en ^{15}N permite así mismo establecer que de forma general poco más del 20% del N de las bacterias adherentes proviene

de la degradación local y el resto del "pool" general de NH_3 del rumen. Ello posibilita resolver la limitación que implica la contaminación microbiana en los estudios *in situ* con alimentos previamente enriquecidos con ^{15}N utilizando el método de dilución (y que imposibilita su adecuada aplicación) al poder así corregir esta contaminación. Hecho que ha sido recientemente demostrado por este equipo (datos no publicados).

CAPITULO 7

CONCLUSIONES

1. El tratamiento combinado con ácidos y calor de la harina de girasol y el guisante de primavera aumenta la fracción de proteína dietaría absorbida en el intestino mediante la reducción de su degradabilidad ruminal, no afectando estos tratamientos la digestibilidad intestinal de ésta.

2. El tratamiento con ácido málico proporciona mejor protección de las proteínas que el correspondiente al ácido ortofosfórico en la harina de girasol. Este aspecto es solamente aparente en la protección del guisante de primavera.

3. Dado que los efectos de la protección son básicamente debidos a la traslación del sitio de digestión del rumen al intestino, la aplicación de estos tratamientos estaría más justificada en alimentos con altos contenidos en proteínas de elevada degradabilidad, limitándose así los efectos de los tratamientos sobre la fermentecibilidad de otras fracciones nutritivas del alimento y consecuentemente sobre la síntesis ruminal de proteína microbiana.

4. La corrección por la contaminación microbiana resulta necesaria para la correcta estimación *in situ* de la fracción de proteína by-pass y de su digestibilidad intestinal en los dos alimentos testados.

5. El guisante de primavera producido en España puede presentar una muy baja digestibilidad intestinal de su proteína by-pass. Resulta, pues, conveniente el control de calidad a este respecto.

6. La existencia de una relación muy estrecha entre los enriquecimientos en ^{15}N de las bacterias adherentes y libres del rumen queda nuevamente demostrada, sin apreciarse variaciones de importancia respecto de la relación previamente obtenida. Ello sugiere la existencia de una ley general relativa a la captación de N degradable en las microcolonias de bacterias adherentes.

7. Esta ecuación permite predecir la subvaloración que se comete en las estimas del aporte de nutrientes por las bacterias adherentes si se utiliza ^{15}N como marcador microbiano y se consideran las bacterias libres como referencia. La consideración de esta subvaloración podría mejorar la precisión de la contribución de la proteína microbiana al flujo de proteína metabolizable, y por tanto, mejorar la precisión de los sistemas de alimentación de rumiantes.

8. Los resultados del estudio de cebo sugieren la posibilidad de disminuir el contenido proteico de dietas concentradas para corderos de crecimiento moderado hasta niveles de 14,5% sobre materia seca utilizando proteínas protegidas. Un aporte adicional de aminoácidos digestibles sobre esta recomendación no mejora los parámetros productivos, ni los rendimientos a la canal.

9. El aumento en la velocidad de crecimiento y en los rendimientos a la canal en los corderos alimentados con concentrados protegidos con ácido málico muestra el interés del uso de este tratamiento en estos piensos, lo que puede ser debido a una reorientación de la fermentación ruminal con una mayor producción de ácido propiónico.

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