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ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
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RUMINAL DEGRADATION OF PROTEIN:
IMPLICATIONS FOR POLLUTING EMISSIONS

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JORGE LEONARDO VANEGAS RUIZ

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Ph.D. THESIS

***RUMINAL DEGRADATION OF PROTEIN:
IMPLICATIONS FOR POLLUTING EMISSIONS***

JORGE LEONARDO VANEGAS RUIZ

Agricultural Engineering

SUPERVISORS

Dr. JAVIER GONZÁLEZ CANO

Dra. M^a. DOLORES CARRO TRAVIESO

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Ph.D. Student

Fdo.: Jorge Leonardo Vanegas Ruiz
Agricultural Engineering

VºBº
Los Directores de la Tesis

Fdo.: D. Javier González Cano
Dr. in Agricultural Engineering

Fdo.: D. Mª. Dolores Carro Travieso
Dr. in Veterinary

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DEDICATION

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ABBREVIATIONS

a	:	Soluble
A	:	Asymptotic Gas Volume
AA	:	Amino acids
ADF	:	Acid Detergent Fibre
AGPR	:	Average Gas Production Rate
ARC	:	Agricultural Research Council
ADIN	:	Acid Detergent Insoluble N
b	:	Non-Soluble Degradable Fraction
CA	:	Casein
CEL	:	Cellulose and starch in proportions 75:25
CH ₄	:	Metane
CP	:	Crude Protein
DMED	:	Dry Matter Effective Degradability
DMD	:	Disappearance of Dry Matter
DM	:	Dry Matter
DMED	:	Dry Matter Effective Degradability
ED	:	Effective Degradability
GHG	:	Greenhouse gas
K _d	:	Fractional Degradation Rate Of B Fraction
NDIN	:	Neutral Detergent Insoluble N
NDF	:	Neutral Detergent Fibre
NH ₃	:	Ammonia

NH ₃ -N	:	Ammonia-Nitrogen
NPN	:	Non-Protein Nitrogen
OM	:	Organic Matter
OMD	:	Organic Matter Disappearance
OMAF	:	Organic Matter Apparently Fermented
r	:	Undegradable Fraction
SP	:	Soybean Protein
SS	:	Sunflower Seed
STAR	:	Starch and cellulose in proportions 75:25
SM	:	Sunflower Meal
VFA	:	Volatile Fatty Acids
WG	:	Wheat Grain
WS	:	Wheat Straw

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RESUMEN

Los rumiantes contribuyen a la emisión de gases de efecto invernadero, principalmente a través del metano (CH_4) producido por la fermentación ruminal, que tiene un efecto invernadero entre 21 y 25 veces más potente que el CO_2 . Por esta razón, en los últimos años se han desarrollado numerosas investigaciones que se han centrado en el análisis de los factores dietéticos que afectan a la producción de este gas, si bien apenas se ha investigado el efecto de las características de las fuentes de nitrógeno. Con este trabajo se pretende, además de estudiar estos efectos, ampliar el conocimiento sobre los alimentos proteicos, estudiando un aspecto poco conocido como es la degradación de los compuestos nitrogenados asociados con las paredes celulares de los alimentos de origen vegetal. La tesis consta de cuatro experimentos:

Experimento 1. El objetivo de este experimento fue analizar la degradación ruminal de las fracciones NDIN y ADIN en muestras de alimentos y comparar los resultados obtenidos con la técnica de dilución del ^{15}N cuando se corrigen para la contribución de las bacterias asociadas a la fase sólida con los valores obtenidos sin corregir. Se utilizaron muestras de semillas de girasol (**SS**), grano de trigo (**WG**) y paja de trigo (**WS**) obtenidas a partir de cultivos fertilizados con ^{15}N para aumentar su abundancia. Estos tres alimentos se seleccionaron debido a que muestran una amplia gama de las fracciones FND, FAD, NDIN y ADIN. Se utilizaron tres ovejas Talaverana provistas con cánulas ruminales, alimentadas con una dieta mixta de heno de alfalfa y concentrado (en relación 2:1 en materia fresca). Las incubaciones ruminales se realizaron con bolsas de nylon que contenían aproximadamente 3 g de cada alimento, que se incubaron en el rumen de cada oveja en dos series de incubaciones durante 4, 24, 48 y 72 horas. A partir de estas incubaciones se determinó la desaparición de materia seca (MS), N-total, FND, FAD, NDIN y ADIN, ajustándose las cinéticas para cada animal a un modelo exponencial simple. Para las fracciones N-total, NDIN y ADIN se

establecieron las cinéticas de degradación con el mismo modelo, corregidas para la contaminación microbiana. Los valores de abundancia en ^{15}N para N-total y NDIN fueron similares, mientras que para ADIN disminuyeron (1,28, 9,95 y 9,24% de reducción en SS, WG y WS, respectivamente). Este hecho sugiere una contaminación con N (a partir del bromuro de cetil-trimetil amonio) en el proceso de aislamiento de la FAD y por tanto una sobrevaloración del ADIN que probablemente sería función de la polaridad de la fibra. Los procedimientos de aislamiento de FND y FAD no eliminan una gran parte de los microorganismos adheridos al alimento durante la incubación ruminal, lo que implica infravalorar la degradabilidad efectiva del NDIN y ADIN si esta contaminación no se considera. Los resultados corregidos por la contaminación microbiana también muestran una contribución considerable del NDIN al N-total degradado (7,25, 13,7 y 20,3% en SS, WG y WS, respectivamente), mientras que para el ADIN solo alcanza cierta importancia en los alimentos fibrosos (2,69, 0,27 y 7,25% en SS, WG y WS, respectivamente).

Experimento 2. El objetivo de este experimento fue analizar la contribución de la fermentación de la proteína a la producción de CH_4 *in vitro*, así como investigar su influencia en la fermentación de dos sustratos de diferente composición. Para ello se utilizó fluido ruminal obtenido de cuatro ovejas fistuladas en el rumen y alimentadas con una dieta mixta 2:1 de heno de alfalfa y un concentrado comercial. Se utilizaron dos sustratos puros con diferente velocidad de fermentación, formados por mezclas de almidón y celulosa en proporciones 75:25 y 25:75 (**STAR** y **CEL**, respectivamente) y tres fuentes de N: NH_4Cl (N no proteico; **NNP**), caseína (**CA**) y proteína de soja (**SP**). Las incubaciones se llevaron a cabo con cultivos discontinuos de microorganismos ruminales para evaluar el efecto de la sustitución (0, 50 y 100%) del NNP por N proteico en la producción *in vitro* de CH_4 y en la fermentación de los sustratos. La cantidad de N aplicada en cada tratamiento fue de 35 mg N/g de materia orgánica de sustrato y las incubaciones duraron 16,5 h. Con ambas proteínas,

se detectaron interacciones fuente N x sustrato ($p = 0,065$ a $0,002$) para la producción de CH_4 y la relación $\text{CH}_4/\text{ácidos grasos volátiles (AGV)}$. Los aumentos en la producción de CH_4 debidos a la sustitución de NNP por N proteico fueron mayores ($P < 0,05$) para el sustrato STAR que con el sustrato CEL; por el contrario, la producción de AGV totales fue mayor con CEL que con STAR. El aumento del nivel de sustitución del NNP por CA o SP dio lugar a variaciones lineales en la relación CH_4/AGV , que aumentó con el sustrato STAR y tendió ($P < 0.10$) a reducirse con el sustrato CEL. La concentración de $\text{NH}_3\text{-N}$ disminuyó con el suministro de cantidades crecientes de ambas proteínas, lo que podría indicar una incorporación directa de aminoácidos y péptidos en la proteína microbiana con una consiguiente reducción de la desaminación. En incubaciones con las fuentes de N como único sustrato, la fermentación de 1 mg de CA o SP produjo 1,24 y 0,60 μmol de CH_4 , respectivamente. Los resultados indican que la generación de CH_4 debido de la fermentación de las proteínas y que puede diferir con el sustrato basal.

Experimento 3. El objetivo de este estudio fue analizar el efecto del tratamiento de la harina de girasol (SM) y la semilla de girasol (SS) con ácido málico y calor a 150°C durante 1 (MAL1) o 3 horas (MAL3) en la fermentación ruminal *in vitro* y la emisión de CH_4 . Se realizaron 2 incubaciones *in vitro* diferentes utilizando fluido ruminal obtenido de 4 ovejas fistuladas en el rumen y alimentadas con una dieta mixta 2:1 de heno de alfalfa y un concentrado comercial. En la primera incubación se evaluó la cinética de producción de gas hasta 144 h y en la segunda se determinaron los principales parámetros de la fermentación a las 16,5 h. El tratamiento MAL1 causó pequeños cambios en los contenidos de PB, FND, FAD y LAD en los dos alimentos, posiblemente debidos al efecto de dilución asociado con la adición de ácido málico en el tratamiento de protección. Este tratamiento no tuvo efectos negativos sobre la producción de gas, pero redujo significativamente los valores de la degradabilidad efectiva de la materia seca en SS (20,3%) y de forma numérica en SM (3,6%)

debido a una moderada ralentización de su fermentación, lo que implicaría un cambio del lugar de digestión del rumen al intestino. Así mismo, este tratamiento fue efectivo en la protección de la proteína, como lo indica la reducción de las concentraciones de $\text{NH}_3\text{-N}$ en SS (26,5%) y SM (14,5%) respecto a las muestras sin tratar. Además, el tratamiento MAL1 no aumentó la concentración de ADIN en SS o SM, lo que indicaría que no produjo daño en sus proteínas. Esta protección de la proteína se asoció con reducciones de la producción de CH_4 (15,5% y 11,3% para SS y SM, respectivamente), en la proporción de CH_4 en el gas producido y en las relaciones CH_4/AGV totales y acetato/propionato, evidenciando todo ello una clara mejora de la eficacia de fermentación. Por el contrario, el tratamiento MAL3 condujo a una sobreprotección, asociada a una importante reducción del valor nutritivo como lo demuestran los incrementos observados en los contenidos en fibra neutro detergente, fibra ácido detergente, lignina, NDIN y ADIN, así como la reducción en la producción de gas, la degradabilidad efectiva y la producción de AGV en comparación con los valores observados en las muestras sin tratar.

Experimento 4. En este experimento se comparó el efecto del tratamiento con ácido málico y calor a 150°C durante 1 h de **SS** y **SM** con el uso como aditivo de la misma dosis de ácido málico o de su sal disódica en la fermentación ruminal *in vitro* y la producción de CH_4 a 6 y 16,5 h. Así mismo se estudió el efecto del tipo de inóculo microbiano, que fue obtenido de las cuatro ovejas fistuladas en el rumen utilizadas en el experimento anterior. Las ovejas recibieron dos dietas mixtas formadas por heno y concentrado en relación 50:50 (**MC**) y por paja de cebada y concentrado en relación 15:85 (**HC**). Estas dietas se consideraron representativas de las utilizadas en rumiantes en lactación y en los sistemas intensivos de producción de carne, respectivamente. El tratamiento de protección se mostró más efectivo que en el experimento anterior pese a haberse utilizado la misma muestra de SS y SM. Así, las reducciones medias de la producción de metano a las 16,5 h fueron 60,3% en SS y 26,7%

en SM y en las concentraciones de $\text{NH}_3\text{-N}$ fueron el 45,3% y 17,2% para SS y SM, respectivamente. Además, se observó también una relación positiva altamente significativa ($P < 0,001$) entre las concentraciones de $\text{NH}_3\text{-N}$ y CH_4 en los dos tiempos de incubación y en ambos alimentos. El tratamiento produjo también otras mejoras de la eficacia de fermentación como la reducción de las relaciones CH_4/AGV totales y acetato/propionato. El uso como aditivo de la misma dosis de ácido málico o su sal sódica presentó algunos efectos positivos en la fermentación, pero éstos fueron más reducidos que los efectos del tratamiento de protección. Para algunos parámetros fermentativos, los efectos del tratamiento de protección fueron más marcados con el inóculo HC que con el inóculo MC.

SUMMARY

Ruminants contribute to the emission of greenhouse gases mainly through the methane (CH_4) produced by rumen fermentation, that has a greenhouse effect between 21 and 25 times more potent than CO_2 . For this reason, in recent years a lot of research has focused on the analysis of the dietary factors affecting the production of CH_4 , although little is known on the effects of the characteristics of the nitrogen sources. In this work some of these effects are studied and additionally the degradation of the nitrogen compounds associated with the cell wall was investigated in three different feeds. The thesis consists of four experiments.

Experiment 1. The aim of this study was to determine the rumen degradation of the NDIN and ADIN fractions in several feeds and to compare the results obtained with the ^{15}N dilution technique either corrected for the contribution of the solid-associated bacteria or uncorrected. Samples of sunflower seed (**SS**), wheat grain (**WG**) and wheat straw (**WS**) obtained from crops fertilized with ^{15}N were used. These three feeds were selected because they show a large range of NDF, ADF, NDIN and ADIN contents. Three Talavera sheep provided with ruminal cannulae were fed a mixed diet of lucerne hay and concentrate (in relation 2:1 on fresh matter). Ruminal incubations were performed with nylon bags containing approximately 3 g of each feed, which were incubated in the rumen of each sheep in two series of incubations for periods of 4, 24, 48 and 72 hours. The DM, total-N, NDF, ADF, NDIN, and ADIN disappearances were determined by adjusting the values for each animal to a simple exponential model. For N-total, NDIN and ADIN fractions, the same model was used and values were corrected for microbial contamination. The values of ^{15}N abundance for total-N and NDIN were similar, whereas those for ADIN were lower (1.28, 9.95 and 9.24% for SS, WG and WS, respectively). This fact suggests a contamination with N (from cetyl trimethyl ammonium bromide) in the process of isolation of the ADF, and therefore an overestimation of ADIN that would probably be function of the polarity of the fibre. The

procedures for NDF and ADF isolation do not remove a large fraction of adherent microorganisms, which results in an underestimation of the effective degradability of NDIN and ADIN when this contamination is not considered. The results corrected for microbial contamination showed a considerable contribution of the NDIN to the total-N degraded (7.25, 13.7 and 20.3% in SS, WG and WS, respectively), whereas for ADIN this contribution had only some importance for fibrous feeds (2.69, 0.27 and 7.25% in SS, WG and WS, respectively).

Experiment 2. The objective of this experiment was to investigate the contribution of protein fermentation to *in vitro* CH₄ production, and to assess its influence on the fermentation of two pure substrates composed by carbohydrates of variable fermentation rate. *In vitro* incubations were carried out with batch cultures of ruminal micro-organisms from sheep fed a 2:1 mixed diet of lucerne hay and a commercial concentrate. The two substrates were mixtures of maize starch and cellulose in proportions of 75:25 and 25:75 (**STAR** and **CEL**, respectively), and the three nitrogen (N) sources were: NH₄Cl (non protein N; **NPN**), casein (**CA**) and isolated soybean protein (**SP**). Five isonitrogenous treatments were made by replacing non protein N (NPN) with CA or SP at levels of 0 (NPN), 50 (CA50 and SP50, respectively) and 100% (CA100 and SP100) of total N. All N treatments were applied at a rate of 35 mg of N/g of substrate organic matter and incubations lasted 16.5 h. With both proteins, N source x substrate interactions ($P = 0.065$ to 0.002) were detected for CH₄ production and CH₄/total volatile fatty acid (VFA) ratio. The increases of CH₄ production observed by replacing the NPN with protein-N were higher ($P < 0.05$) for STAR than for CEL substrate, but the opposite was observed for the increases in VFA production. As a consequence, replacing the NPN by increased levels of CA or SP led to linear increases of CH₄/total VFA ratio with STAR, whereas CH₄/total VFA ratio tended ($P < 0.10$) to be decreased with CEL substrate. Increasing the amount of both proteins decreased linearly ammonia-N concentrations, which

may indicate an incorporation of amino acids and peptides into microbial protein without being deaminated into ammonia-N. In incubations with the tested N sources as the only substrate, the fermentation of one mg of CA or SP produced 1.24 and 0.60 μmol of CH_4 , respectively. The results indicate the generation of CH_4 from protein fermentation may differ with the basal substrate.

Experiment 3. The objective of this study was to analyse the effects of the treatment of sunflower meal (**SM**) and sunflower seed (**SS**) with malic acid and heat at 150°C for 1 (**MAL1**) or 3 hours (**MAL3**) on *in vitro* ruminal fermentation and CH_4 emission. Two different incubation trials were carried out using ruminal fluid from four rumen-fistulated sheep fed a mixed diet 2:1 of lucerne hay and a commercial concentrate. In the first incubation trial the gas production kinetics was assessed over a 144 h period, and in the second trial the main fermentation parameters were determined after 16.5 h incubation. The MAL1 treatment caused minor changes in the chemical composition of both feeds and had no negative effects on gas production parameters compared with the untreated SS and SM, but reduced the estimates of DM effective degradability (DMED) by 20.3 and 3.6% for SS and SM, respectively. Moreover, this treatment was effective in reducing protein degradation, as indicated by the reduced $\text{NH}_3\text{-N}$ concentrations (by 26.5 and 14.5% for SS and SM, respectively) compared with the untreated samples. However, the MAL1 treatment did not increase the concentration of ADIN in SS or SM, indicating that it did not produce protein damage. The protein protection was associated with reductions in CH_4 production (15.5 and 11.3% for SS and SM, respectively), the proportion of CH_4 in the gas produced, and in the ratios $\text{CH}_4/\text{total VFA}$ and acetate/propionate ratio, thus showing a clear improvement of the efficiency of fermentation. On the contrary, the MAL3 treatment led to an overprotection associated to an important reduction of the nutritive value, as shown by the augmented neutral detergent fibre, acid detergent fibre, lignin, NDIN and ADIN contents, as well as the strong

reductions in the gas production values, DMED effective degradability and VFA production compared with those in the untreated samples.

Experiment 4. The aim of this study was to compare the in vitro fermentation of malic acid-heat (150°C for 1 h) treated **SS** and **SM** with that of the untreated feeds but supplemented with malic acid or disodium malate at 6 and 16.5 h of incubation. Moreover, the influence of the type of inoculum was investigated by using ruminal fluid from four rumen-cannulated sheep and fed either a 50:50 hay:concentrate diet (MC) or a 15:85 barley straw:concentrate (HC) diet. The diets were representative of those for medium-lactation animals and fattening ruminants under intensive systems of production, respectively. The protective treatment was more effective than that observed in the previous experiment, although the same SS and SM samples were used. The average reductions in CH₄ production at 16.5 h incubation were 60.3 and 26.7% for SS and SM, respectively, and in the NH₃-N concentrations were 45.3 and 17.2%, respectively. Moreover, a highly significant ($P < 0.001$) positive relationship was observed between the concentrations of NH₃-N and CH₄ at both incubation times for both feeds. The treatment also resulted in a reduction of the ratios CH₄/total VFA and acetate/propionate. The use of the same dose of malic acid and its disodium salt as additive had some positive influence on fermentation, but the effects were more reduced compared with those of the malic acid-heat treatment. The effects of the malic acid-heat treatment were more pronounced with the HC inoculum than with the MC inoculum for some fermentative parameters.

CHAPTER 1.-

GENERAL INTRODUCTION AND OBJECTIVES

1.1. GENERAL INTRODUCTION

The protection of the environment is currently one of the priorities of the livestock farming in the European Union. The increase in livestock production produced in recent decades has been linked to a modernization of farms, which led to the progressive replacement of traditional farms by highly intensive farming. These changes led initially to improved efficiency in both use of resources and livestock production and, but they can also result in potential risks for the environment mainly due to a high concentration of intensive farms. The most important environmental problems are related to the contamination of waters and soils and to the contribution to global warming through the emission of greenhouse gases (GHG), mainly carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O). Each of these gases has different impacts on the atmosphere, according to its greenhouse power and lifetime.

Livestock farms contribute to the emission of these GHG mainly through two processes: the enteric fermentation that generates CH₄, and the anaerobic fermentation of manure that produces CH₄ and N₂O. Among the different livestock species, ruminants are the main contributors to total CH₄ production, due mainly to the CH₄ produced in the rumen fermentation. The CH₄ is also an energy loss for the animal, representing between 2 to 12% of the gross energy intake. Therefore, reducing the CH₄ production by ruminants is currently being the target of numerous investigations.

1.2. CONTRIBUTION OF LIVESTOCK TO POLLUTING EMISSIONS. THE ROLE OF RUMINANTS

The concern on the emissions of GHG due to their contribution to the negative effects caused by climate change has increased in recent years. Some reports (Steinfeld *et al.*, 2006) pointed out to livestock as one of the main contributors to GHG emissions. Thus, this activity generates CO₂ with an atmospheric lifetime of between 50 and 200 years, CH₄, the second

gas of importance with a time of permanence in the atmosphere between 10 and 20 years, and N₂O with an atmospheric lifetime of 100 to 150 years and responsible for a sharp increase in the radiation modifying the climate (Blas *et al.*, 2008; Dachraoui, 2015). Although CH₄ and N₂O are found in concentrations much lower than the CO₂ in the atmosphere (200 times in the case of CH₄), their contribution to the greenhouse effect reach significant levels, about 18 and 6%, respectively, compared to the 49% attributed to the CO₂ (Rhode, 1990). It is estimated that the livestock sector accounts for 18-20% of total GHG emissions of anthropogenic origin (Steinfeld and Hoffman, 2008; Gill *et al.*, 2010), varying the figures among countries. The contribution of livestock to the generation of these gases is unequal, with estimated values of 9%, 37% and 65% of total emissions for CO₂, CH₄ and N₂O, respectively (Khalil, 2000; Steinfeld and Hoffman, 2008). Livestock farms emit CH₄ and N₂O through two processes: the enteric fermentation, which generated CH₄, and the anaerobic fermentation of the manure, which generates CH₄ and N₂O. Therefore, the concerns about GHG emissions in the livestock sector have focused on both CH₄ and N₂O.

The CH₄ emissions of anthropogenic origin represent about 60% of total CH₄ produced worldwide, whereas natural sources (marshes, oceans, freshwater streams, sediment and wildlife) represent the remaining 40% (Lelieveld *et al.*, 1998; WWF, 2013). The main anthropogenic sources of CH₄ are the treatment and disposal of wastes (landfills), processing of energy (mines, oil, natural gas), industrial processes and agriculture. The agriculture represents on average 51% of anthropogenic emissions and about 31% of total CH₄ produced in the world. The CH₄ production in Spain in 2009 was 18.7 million tons of CO₂-equivalent units, of which 51.5% corresponded to the farming activity, 41.2% to the treatment and disposal of wastes, 7.3% to energy conversion, and 0.1% to industrial processes (MAGRAMA, 2010; WWF, 2013). These figures identify the agricultural activity as the main source of CH₄ in our country, including the emissions from enteric fermentation in

animals (34.4% of total CH₄ produced), manure management (15.4%), rice fields (0.7%) and the burning of agricultural wastes (1.8%).

Therefore, livestock contributes almost to half of the CH₄ produced in Spain, the enteric fermentation representing approximately 2/3 of the total. The herbivores are the animals that contribute most to the CH₄ generated in the enteric processes, and within this group the ruminants are the most important. In ruminants, 85-95% of total CH₄ emission is generated in the enteric fermentation and only a small part (5-15%) is generated by the fermentation of the manure produced (de Blas *et al.*, 2008; Hook *et al.*, 2010; Kumar *et al.*, 2014). The CH₄ is generated in the rumen by a population of methanogenic archaea, which uses as substrates mainly CO₂ and hydrogen (Hook *et al.*, 2010; Kumar *et al.*, 2014). The archaea constitute an important group of microorganisms that contributes to maintain the concentration of hydrogen in the rumen at adequate levels to allow the growth of other microbial species, thus allowing a more efficient fermentation process (Hook *et al.*, 2010). However, CH₄ represents an energy loss for the host animal and contributes significantly to the greenhouse effect and climate change (O'Kelly and Spiers, 1992).

The CH₄ emission rate is low in non-ruminant herbivores (i.e. horses, mules, donkeys) and even lower in omnivores (i.e. pigs). In these animal species the fermentation of nutrients takes place in the cecum and colon, but only the fraction that is not digested in the small intestine (mainly fibre constituents) enters these sections of the digestive tract; moreover, the retention time of digesta is smaller than that in the rumen (de Blas *et al.*, 2008).

The N₂O is formed in the processes of nitrification and denitrification produced by the microorganisms. Therefore, their generation is inversely associated with the efficiency of use of N by the animal and it can be reduced by decreasing the dietary protein in excess of animal requirements (Rotz *et al.*, 1999). Because a great part of the N emissions are due to ammonia losses from the rumen, as a result of an excess of rumen degradable protein, the control of

excesses in this fraction should also reduce these emissions. Grazing animals produce important N₂O emissions, and the excretion of high amounts of N has been shown to cause odors problems and water pollution (Oenema *et al.*, 1997).

1.3. RUMINAL FERMENTATION

Ruminants have a great importance worldwide, but this importance is even more marked in less-favoured regions due to their ability to utilize resources not used by other animals, including low quality pastures. This ability is due to the degradation of plant cell walls by the symbiotic microbial population of the reticulum-rumen and the absorption of fermentation products by the host animal (Rotger, 2004). In Spain, the main exploited species of ruminants are cattle, sheep and goats, but globally there are also other species of relevance (Van Soest, 1994).

The hydrolysis in the rumen of carbohydrates in feed, both structural (constituents of the plant cell wall) and non-structural (mainly starches and sugars) results in the release of monosaccharides, that are subsequently fermented to volatile fatty acids (VFA), lactate and gases (CO₂ and CH₄) as main final products (Figure 1). The VFA are mainly absorbed through the rumen wall and in a smaller proportion in subsequent sections of the digestive system. The acetate goes mostly into the portal circulation, about 20% being captured by the liver and the rest going into the general circulation and used by other tissues. Almost all propionate (95%) that enters the portal circulation is captured by the liver. The absorbed butyrate is converted almost entirely in β -hydroxybutyrate when passing through the ruminal mucosa and the small amount of remaining butyrate enters into the portal circulation (Relling and Mattioli, 2003).

Figure 1. Scheme of ruminal fermentation of polysaccharides and their final products (taken from Lopez, 2009).

The nitrogenous compounds in feeds can be degraded to different extents by ruminal microorganisms and the resulting products can be used to synthesize microbial protein. The main final products of this process are the microbial protein (proteins, but also other N-containing compounds such as nucleic acids, aminosugars, etc.), undegraded feed protein and ammonia (NH₃). The undegraded feed protein and the microbial protein that leave the rumen are subsequently digested, freeing aminoacids (AA) which will be absorbed and transported by the blood circulation to the liver and body tissues. The NH₃ has a dual function: being used for microbial protein synthesis and being absorbed through the wall of the rumen and transported to liver, where it becomes to urea through the urea cycle (Van Soest, 1994). Part of this urea can be recycled to the rumen, either with the saliva or directly through the rumen wall, but most of the urea is excreted in the urine. This excretion is a nitrogen loss and contributes to environmental pollution of livestock farming.

Regarding lipids, the triacylglycerides in feeds can be hydrolyzed by the lipases of the ruminal microorganisms, producing glycerol and free fatty acids (Van Soest, 1994). The glycerol is rapidly fermented to VFA whereas the fatty acids can be used by ruminal microorganisms to form their structures (microbial lipids) or be hydrogenated by the ruminal bacteria. Thus, the duodenal digesta flow contains a mixture of microbial lipids and non-esterified fatty acids, of which some are unsaturated. As already indicated, many of the final products of the fermentation process are used by the host animal (Øskov, 1992; Lopez *et al.*, 2011), although compounds such as CH₄, CO₂ and the excess of ammonia cannot be used and contribute to environmental pollution. Most of the CH₄ produced by the ruminants in the enteric fermentation is generated in the rumen (85-95%) and only a 5-15% is produced in the posterior digestive tract. The CH₄ is expelled outside mainly by belching. Methane is also from the anaerobic fermentation of manure. Crutzen *et al.* (1986) estimated a total production of CH₄ of 600, 276 and 32 liters/day for dairy cattle, beef cattle and sheep, respectively, of which 70-80% was generated in the enteric fermentation and the rest in the fermentation of manure. The energy loss associated with the CH₄ production in ruminants can represent 2-5% of gross energy ingested for diets of high nutritive value and up to 7-12% in animals fed low-quality forages (Crutzen *et al.*, 1986; O'Kelly and Spiers, 1992; McCaughey *et al.*, 1997; Carmona *et al.*, 2005).

1.4. FACTORS AFFECTING THE PRODUCTION OF CH₄ IN RUMINANTS

The amount of CH₄ produced by the ruminants depends on numerous factors that can be grouped into factors related to the animal and those related to the diet.

1.4.1. Animal factors influencing CH₄ production

Animal species, weight, age and production levels are the most important animal factors influencing CH₄ production (de Blas *et al.*, 2008). Cattle produce more CH₄ than sheep and goats, due to its larger size, and CH₄ production increases with increasing age and

weight of the animals, as the rumen develops and the fermentative processes increase as the animals grow (Crutzen *et al.*, 1986; Johnson and Johnson, 1995). Machmüller and Clark (2006) measured the CH₄ production in ruminants (cattle and sheep of both sexes) under grazing conditions in New Zealand and observed that daily CH₄ production increased linearly with the age and weight of the animals, but no differences due to sex were detected. It has been observed that the increase in the intake level (associated with increased production levels) causes a greater rate of passage of feed through the rumen and reduces the time for microbial fermentation, resulting in lower CH₄ production (Blaxter and Clapperton, 1965).

1.4.2. Dietary factors influencing CH₄ production

The diet is one of the main determinants of the amount of CH₄ produced. The factors associated with the diet are variable, as feed characteristics not only affect the nutritive value of the diet, but also the level of intake and eating pattern. These factors influence the ruminal environment and determine the microbial populations growing, as well as the digesta passage rate and therefore the time that feed is available to ruminal microorganisms for fermentation.

One of the main factors influencing CH₄ production is the forage/concentrate ratio in the diet, as it largely determines the ruminal conditions in which the microbial populations develop. Forage diets favor the establishment of fibrolytic flora and *Butyrivibrio* spp., whereas the concentrate-rich diets promote the development of amylolytic flora, with a predominance of *Selenomonas*, *Peptostreptococci* and *Lactobacilli* (Lopez, 2009). In general, as the forage/concentrate ratio in the diet is reduced, there is a reduction in both the acetate/propionate ratio and the amount of CH₄ per unit of organic matter fermented (Johnson and Johnson, 1995; González and Ruiz, 2001; Boadi *et al.*, 2004). In addition, the influence of forage/concentrate ratio of diet on CH₄ production occurs through concomitant changes in pH and rumen microbial populations (Van Soest, 1994; Bonilla and Flores, 2011). The fermentation of fibrous carbohydrates generally results in a high production of CH₄ and high

acetate/propionate ratios (Johnson and Johnson, 1995; Marín, 2013). Conversely, starch-rich diets favor the production of propionate and the amount of CH₄ produced per unit of organic matter fermented is lower than that observed for fibrous diets. Additionally, starch-rich diets produce a decrease in ruminal pH that can potentially cause a reduction in the population of protozoa and contribute to reduced CH₄ production, as some methanogenic archaea are associated with protozoa (Van Soest, 1994; Santacoloma, 2011; Willen, 2011).

The type of forage and its processing are also factors affecting the CH₄ production by ruminants. As the nutritive value of forage increases, the CH₄ production per unit of fermented forage is reduced and energy losses are lower (Blümmel *et al.*, 1997; Carmona *et al.*, 2005; Moss and Givens, 2000). Conversely, the administration of mature or highly lignified forages causes an increase in CH₄ production. In grazing animals the quality of the ingested feed can vary during the year due to climatic factors, so that there are seasonal variations in CH₄ emissions, which are also affected by the supplementation with grains and dried forages done at certain times grazing (González and Ruiz, 2001). It has been also observed that animals fed legume-based diets have lower CH₄ production than those receiving grass-based diets (Hironaka *et al.*, 1996; Mathinson *et al.*, 1998). Moss *et al.* (1994) observed that sheep fed cereal straw treated with NH₃ or NaOH had increased daily CH₄ production as compared with sheep fed untreated cereal straw, but the amount of CH₄ was lower when the CH₄ production was expressed per unit of digestible organic matter intake. Thus, the treatments that improve the nutritional value of low-quality forages can be a useful strategy to reduce the CH₄ production in ruminants fed this type of forages.

The forage processing is one prominent factor influencing the production of CH₄ (Carmona *et al.*, 2005). Forage processing can increase its degradability due to the greater exposure to rumen microorganisms (Thompson and Beever, 1980), but can also result in a decrease of rumen fermentation accompanied by a greater intestinal digestibility as a result of

a change in the place of digestion (García, 1991). García (1991) analyzed the effects of processing on different feeds, and observed that grounding and pelleting forages reduced the degradation rate of DM, crude protein and fibre as consequence of low levels of rumination, and also reduced the rumen retention time of fine particles for both forage and concentrates. Hironaka *et al.* (1996) measured the CH₄ production in steers fed alfalfa hay, either chopped or pelleted, and observed that CH₄ production per kg of DM intake was reduced by 10% when the steers were fed the pelleted with alfalfa hay.

Kurihara *et al.* (1999) measured the CH₄ production in fattening calves fed different diets and observed a curvilinear relationship between the CH₄ production and the gain of weight (Figure 2), resulting in lower CH₄ production for diets that allowed high weight gains. In addition, they found a positive lineal relationship between CH₄ production and the feed conversion rate, indicating that the administration of high-quality diets is a practical means to reduce CH₄ emissions.

Figure 2. Relationship between the CH₄ production and either weight gain (a) or feed:weight gain ratio (b) in animals fed only forage (●) or a diet with a high content in cereal grains (○). Taken from Kurihara *et al.* (1999).

The characteristics of the concentrate can also affect the CH₄ production. Beauchemin and McGinn (2005) observed that CH₄ production in fattening steers was lower with corn-based diets than with diets based on barley, which was attributed to the lower ruminal degradability of the corn, as corn has less floury endosperm than barley grain. Conversely, López *et al.* (2011) in Murciano-Granadina goats observed the opposite effect. The controversial results obtained in different studies might be due to the interactions of cereal grains with other dietary components, especially with protein concentrates.

Only few studies have examined the effect of the protein concentration in the diet on CH₄ emissions, although some *in vitro* experiments have shown that protein degradation generates less CH₄ than carbohydrates degradation (Cone and Van Gelder, 1999; Jentsch *et al.*, 2007). However, protein supplementation can result in increased CH₄ production with N-deficient diets (Carro *et al.*, 1999a; Kurihara *et al.*, 1999; Kirchgessner *et al.*, 1991; Singh *et al.*, 2011), that has been attributed to an increased fibre degradation and microbial growth (Ranilla *et al.*, 2001; Cone *et al.*, 2009). On the contrary, Tamminga (1992) observed a reduction in CH₄ emissions (expressed as proportion of the digestible energy intake) by increasing the N content of the grass, that was attributed to the lower fibre content of the grasses with higher N content. These results indicate the difficulty of studying the contribution of dietary protein to CH₄ production, due to the complex protein-carbohydrates interactions. Moreover, the type of N supplemented can also affect the production of CH₄. Carro and Miller (1999) observed that the partial substitution (50%) of NPN by amino acids, peptides or soybean protein resulted in a significant increase in the amount of CH₄ produced in Rusitec fermenters that received a fibrous substrate. This study also showed that the fermenters supplemented with amino acids produced a higher amount of CH₄ compared with those supplemented with isonitrogenous amounts of peptides or protein.

Another factor influencing the CH₄ production is the addition of fats to the diet of ruminants. Johnson and Johnson (1995) observed a decrease in CH₄ emissions by increasing the amount of fat as long as the amount of added fat did not adversely affect the rumen fermentation. The addition of small amounts of long-chain unsaturated fatty acids present in oil seeds (i.e. cotton, soybean, sunflower) caused a decrease both in the CH₄ emission and in the generation of glucogenic precursors (Santacoloma, 2011). This reduction in the CH₄ production occurs through biohydrogenation of the unsaturated fatty acids, thereby reducing the hydrogen available for the formation of CH₄, but also through a direct negative effect of

the polyunsaturated fatty acids on the *methanogenic archaea* (Hindrichsen *et al.*, 2006). Moreover, the adhesion of free fatty acids to feed particles reduces microbial colonization and fermentation, which also contributes to reduce the production of CH₄ (Van Soest, 1994). Dong *et al.* (1997) analyzed the effect of the inclusion of different types of oil (canola, cod liver and coconut) in the diet of fattening steers and observed that the effects on CH₄ production depended not only on the type of oil, but also on the basal diet, the reduction being greater for diets containing concentrates compared with only forage diets. The depression in CH₄ production tends to increase with the degree of unsaturation of fatty acids, although the decrease in CH₄ production induced by unsaturated fatty acids is not directly proportional to the number of double bonds (Dong *et al.*, 1997).

Another dietary factor affecting CH₄ production is the use of additives. One of the most effective additives are the ionophores, which appear to reduce the population of methanogenic archaea and the protozoa associated with them (Beauchemin and McGinn, 2006a). However, these additives are banned in the European Union since 2006 and their use in other countries is being widely questioned. Other additives which can reduce CH₄ production, are some organic acids (i.e. fumaric acid, malic acid; Carro and Ranilla, 2003a, 2003b; Giraldo *et al.*, 2007), some essential oils (Beauchemin and McGinn, 2006b; Calsamiglia *et al.*, 2007), garlic compounds (Busquet *et al.*, 2005; Kamel *et al.*, 2008) and secondary compounds of plants such as tannins (Animut *et al.*, 2008). However, so far none of them is effective enough to be use in the practical feeding of ruminants.

1.5. DEGRADATION OF NITROGENOUS COMPOUNDS

The protein in ruminant diets has a double objective: to meet the needs of nitrogen of the ruminal microorganisms and to provide AA for the host animal. Unlike monogastrics, the quantity and the profile of the AA reaching the duodenum is different to those provided in the diet, due to protein degradation and microbial protein synthesis. The AA needs of the host

animal can be met by AA from dietary or microbial origin, whereas the rumen microorganisms can use both protein N and NPN. The supply of NPN (nucleic acids, small peptides, free amino acids, urea, amides, amines, ammonia, ...) to the rumen is done by the feed and by the recycling of endogenous urea that can enter the rumen via saliva or by passive diffusion through the ruminal epithelium (Hegarty and Peterson, 1973). Among the dietary contributions, the true protein is the most important fraction, whereas the contribution of NPN compounds is minor and depends on the type of feed.

The protein ingested by the ruminants undergoes an extensive degradation by the rumen microorganisms, which produce the necessary enzymes to hydrolyze the peptide bonds to generate peptides, oligopeptides and AA (Figure 3). Peptides and AA can be used directly by ruminal microorganisms for microbial protein synthesis or being deaminated to produce NH_3 and carbon skeletons. The NH_3 is the main source of N for ruminal microorganisms, providing between 40 and 100% of the N requirements for microbial protein synthesis (Stern and Hoover, 1979; Wallace and Cotta, 1988; NRC, 2001).

Figure 3. Scheme of protein fermentation in the rumen (taken from Van Lier and Regueiro, 2008).

The ciliated protozoa are able of ingesting small feed particles, as well as bacteria and fungi, and proteolysis occurs inside the cell (Tamminga, 1979). However, most of the rumen proteolytic activity is due to the bacteria and this activity is also primarily responsible for the recycling of bacterial protein.

1.5.1. Factors affecting protein degradation in the rumen

Several authors (Stern and Hoover, 1979; Tamminga, 1979; Ørskov and McDonald, 1979; Wallace, 1996) indicate that the degree of protein degradation depends on the amount of degradable protein, the degradation rate and the residence time in the rumen. Thus, the multiple factors affecting the protein degradation can be divided in two groups: I) the factors that determine the susceptibility of protein to microbial degradation which are related to feed characteristics, including feed processing, and II) those affecting the intensity and extent of microbial activity, which are influenced by the digestive physiology of the animal and diet characteristics (i.e. intake level, granulometry, forage/concentrate ratio, ..) that determine the rumen conditions such as the pH, the supply of nutrients to the microorganisms and the rate of passage of digesta (Arroyo, 2012).

Within the first group of factors, the solubility of the protein has a great importance, as in general the more soluble, the more accessible to microbial enzymes, although there are some exceptions. It has been long assumed (Chalmers and Synge, 1954; NRC, 2001) that protein degradation is influenced by its solubility in the rumen fluid, because soluble compounds are degraded faster due to its easily access by microorganisms. However, there are differences between the true protein and the NPN compounds (Gargallo, 2006), as the NPN compounds are rapidly degraded in the rumen ($> 300\%/h$) and have degradability values of 100% (Sniffen *et al.*, 1992). There is controversy on the use of the solubility of the protein as a predictor of its ruminal degradability. Thus, several authors (Mahadevan *et al.*, 1980; Stern and Satter, 1984) indicate that there is no direct and simple relationship between

the solubility of the protein and its rumen degradability. The correlation between protein solubility and the values of ruminal degradability is generally low, although the solubility values can be used to compare the ruminal degradability of samples of the same protein supplement. A lack of relationship between solubility and degradability is usually obtained by trying to obtain a linear relationship between the two parameters, but better adjustments are obtained with parabolic quadratic models. Thus, for feeds with protein of low solubility and degradability (i.e. meat meal, fish meal) both parameters are linearly related (Madsen and Hvelplund, 1987; González *et al.*, 1998), whereas for feeds with protein of high solubility the values are located in the asymptote of the parabolic model. Madsen and Hvelplund (1987) reported a determination coefficient of 0.64 for 118 samples of concentrates using a parabolic model to relate solubility and degradability, but the relationship can be improved when less variable feeds are considered. Thus, Andrés (1990) obtained a determination coefficient of 0.71 for 62 samples of protein concentrates, and the prediction was improved when the samples of soybean meal and soybean seed were excluded ($R^2 = 0.81$; $n = 53$).

The type of protein plays an important role in its ruminal degradation. Thus, prolamins and glutelins are insoluble and have slow degradation rates, whereas globulins are highly soluble and degradable (Romagnolo *et al.*, 1994). The type of protein is associated with other characteristics such as its three-dimensional structure, with intra and intermolecular bonds that impede the access of the proteolytic enzymes and the presence of disulphide bonds or bonds within or between chains of proteins (tertiary and quaternary structures). In addition, peptide bonds as those joining L-proline and methionine-proline are more resistant to degradation than others (Wallace and Kopêcný, 1983; Yang and Russell, 1992). Other factors that can reduce or inhibit proteolysis are the physical barriers that limit the access of bacteria (i.e. cell walls) and anti-nutritive factors (Sniffen *et al.*, 1992; NRC, 2001; Bach *et al.*, 2005). Furthermore, any treatment modifying the protein structure (tertiary

or quaternary) can result in protein denaturation or cause the reaction of protein with other compounds, preventing the proteolytic action of microbial enzymes. The most widely used treatments to protect the protein against rumen degradation are the treatment with heat and acids.

In general, it is assumed that the ruminal degradation of a protein is inversely related to the rate of passage of digesta through the rumen (Ørskov and McDonald, 1979). However, this assumption does not take into account that increasing the rate of passage also increases the proteolytic activity, causing an increase in the rate of degradation (González *et al.*, 1987; Ørskov, 1992). The passage rate is influenced by different dietary factors, such as the intake level, frequency of feeding, forage/concentrate ratio and feed particle size, among others. The level of intake is probably the factor that most influences the rumen rate of passage of both liquid and feed particles. The rumen is a continuous flow system, where the amount of feed and fluid leaving the system (by degradation, absorption and transit) is similar to that entering, and therefore increasing the intake level usually increases the rate of passage (Tamminga, 1979; Hoover and Miller, 1992; Ørskov, 1992). Thus, an increase in the rate of passage through the rumen will decrease the digesta retention time and therefore the time that feed is available for microbial degradation, resulting in reduced estimations of effective degradability. However, a high intake level is also associated with an increase in the rate of degradation, resulting in at least a partial compensation of both effects on the estimations of the effective degradability (González *et al.*, 1987; Rodríguez *et al.*, 2008). Regarding this point, a positive correlation between the rate of evacuation of particles from the rumen and the protein degradation rate has been observed for both concentrates and forages.

The forage/concentrate ratio of the diet has an effect on the rate of evacuation of the rumen as it influences the quantity of forage ingested, ruminal motility, salivation and the opening rate of the reticulum-omasum orifice (Evans, 1981; Ørskov, 1992). Thus, decreasing

the forage/concentrate ratio generally reduces the rumen evacuation rate due to the reduction in rumination, ruminal motility, opening rate of the reticulum-omasum orifice, as well as shifts in the ruminal populations caused by the changes in the nutrients available and the production of saliva. Poncet *et al.* (1987) observed that reducing forage/concentrate ratio in the diet caused a decrease in the rumen evacuation rate which was associated with reductions in the rate of protein degradation, thus indicating a compensation of both effects on the estimation of the protein effective degradability (González *et al.*, 1987). When diet was supplied at 1.1 energy maintenance, increasing the proportion of concentrate from 10 to 60%, this compensation was only partial, resulting in a decrease of the effective degradability; however, when the diet was fed *ad libitum* increasing the proportion of concentrate from 30 to 60% (the diet with 10% concentrate was not tested) the compensation was total and the estimations of the effective degradability were not modified.

Faría-Mármol *et al.* (2002) tested four alfalfa samples (two green, one hay and one dehydrated) in diets consisting in alfalfa hay and concentrate in ratios of 2/1 and 1/2, and also observed a reduction in the rate of protein degradation by decreasing the forage/concentrate ratio. The forage/concentrate ratio may have effects on the dominant microbial populations in the rumen and therefore on the fibrolytic activity, which can have an impact on feed degradability. However, the effect of forage/concentrate ratio on the ruminal populations and the rate of passage through the rumen is difficult to understand, as other factors (i.e. the intake level) are also involved (Huntington and Givens, 1995).

The rumen pH is an essential parameter in the rumen degradation processes and ruminant nutrition. Decreases in rumen pH are usually associated with decreases in protein degradability. However, the optimal pH for proteolytic enzymes varies from 5.5 to 7.0 and these values are within the physiological range of rumen pH (Kopečný and Wallace, 1982), so that the decrease in proteolysis by low pH cannot be attributed to enzyme inhibition. Low

pH values (< 5.5) lead to a drastic decrease of some bacterial strains capable of degrading peptides and AA deamination. The pH decrease is generally associated the intake of high amounts of starch or other sources of rapidly-fermentable carbohydrates (Owen *et al.*, 1998). Increased intake level is also associated with a reduction in pH by increasing the amount of acids derived from fermentation, leading to an increase in passage and degradation rates and influencing the degradation process. Shiver *et al.* (1986) showed that the reduction of bacterial populations caused by low pH values can be due to difficulties of adhesion of bacteria to the substrate, as well as to reductions in the rumen retention time.

There are multiple dietary factors that determine the ruminal pH. Cardozo *et al.* (2002) observed a low degradability of the protein in high-concentrate diets in continuous fermenters regardless of pH values. It is worth to remember that increasing the intake level usually leads to a drop in pH and increases in the rate of degradation as already discussed before. The influence of the rumen pH and the type of diet on protein degradability is due to complex interactions among many factors.

1.6. PROTECTION OF PROTEIN FROM RUMINAL DEGRADATION. IMPLICATIONS AND METHODS

The main objective of protein feeding in ruminants must be getting the maximum N efficiency to maximize animal production per unit of N consumed. However, ruminants are inefficient at transforming dietary N in milk-N or meat-N, as only 20 to 30% of ingested N is retained for milk production or growth and the rest of the ingested N is excreted in the feces and urine (Tamminga *et al.*, 1992; Bequette *et al.*, 2003). One of the main factors involved in this low efficiency is the high proportion of degradable N in most feeds for ruminants, which is associated with important leakages of $\text{NH}_3\text{-N}$ in the rumen. Feeding an excess of degradable protein has a negative impact on the energy balance of the animal, due to the energy cost of transforming $\text{NH}_3\text{-N}$ into urea in the liver, but has also negative environmental

consequences due to the N emissions and the generation of GHG (Arroyo, 2012). Moreover, the increasing environmental concern about livestock production makes the reduction of the emissions a priority in many countries, especially in the European Union, thus increasing the need of improving the efficiency of N use in ruminants. Therefore, there are many reasons to reduce the protein degradation in the rumen and shift the protein digestion to the small intestine.

The deamination of the AA resulting from protein degradation produces NH_3 and carbon skeletons that are fermented by ruminal microorganisms to VFA and can contribute to CH_4 generation. Several studies (Carro and Miller, 1999; Carro *et al.*, 1999c) have indicated that the fermentation of these carbon skeletons can contribute to the supply of hydrogen to form CH_4 , and that decreasing the protein degradation could result in lower CH_4 production. However, only few studies have addressed the relationship between protein degradation and CH_4 production.

A suitable strategy to reduce the overall degradation of protein in the rumen is feeding high-protein feeds with low degradability, such as meat meal, fish meal and gluten meal; however, both meat and fish meal are banned for ruminants feeding in the European Union and the strong deficit of lysine in gluten meal is a disadvantage. Taking into account these limitations, the treatment of high-protein feeds to reduce their ruminal degradability and increase their content in protein digestible in the intestine has a great interest, not only nutritional, but also economic and environmental. There are different methods to decrease the ruminal fermentation of protein, which can be classified into physical and chemical treatments (Windschitl and Stern, 1988; NRC, 2001). Most of these methods are based on the application of heat, chemical agents or a combination of both for modifying the characteristics of the protein and increasing their resistance to microbial degradation (Broderick *et al.*, 1991).

Most of physical treatments involve the use of heat and these methods were first described by Chalmers *et al.* (1954). The heat treatment is one of the most used procedures because of its effectiveness, profitability, safety and ease of application. However, heat effects can be highly variable, varying from beneficial protective effects to protein damage. The mechanism of action of heat is based on the protein denaturation that increases the resistance of protein to microbial proteolytic enzymes. However, if the thermal impact is excessive Maillard irreversible condensation reactions are produced between the free amino groups of protein and the aldehyde groups of sugars (Broderick *et al.*, 1991; NRC, 2001; Arroyo, 2012). These irreversible reactions cause significant losses of certain AA and decrease protein digestibility in the small intestine, thus generating "overprotected proteins" (Van Soest, 1994). Numerous methods of applying heat to feeds have been tested in the practical feeding, such as toasted, extrusion, expeller meal and microwave. All of them have shown their effectiveness to decrease ruminal degradability of the main protein concentrates used for ruminant feeding including soybean meal, cottonseed meal, rapeseed meal and sunflower meal. The effectiveness of the heat treatment depends on the time and temperature applied, but the optimal conditions vary with the protein concentrate (Broderick *et al.*, 1991; McNiven *et al.*, 2002). Thus, Calsamiglia and Stern (1995) observed a decrease of intestinal digestibility by heating the soybean meal at 165°C for more than 2.5 h, whereas milder heating resulted in an increase of protein digestibility. Satter (1986) observed similar levels of protection by treating whole cottonseed either at 160°C for 20 minutes or at 140°C for 60 minutes, but when the heat exposure was less than 12 minutes higher temperatures (180°C) were required for an effective protection.

The treatment of protein concentrates to reduce the rumen degradability of protein can have a positive effect on ruminant performance. Hsu and Satter (1995) reported increases in milk production of dairy cows by feeding heat-treated soybean meal and other studies with

similar results have been reviewed by Santos *et al.* (1998). Broderick *et al.* (1990) observed that feeding expeller soybean meal enhanced milk production compared with the feeding soybean meal obtained by solvent (32.7 vs. 32.1 kg/day, respectively). Similarly, Titgemeyer and Shirley (1997) observed a 4% of increase in the efficiency of production in dairy cows by feeding expeller soybean meal with no negative effect on intake. The effectiveness of the expeller meal can be attributed to the heat generated during the pressing process, which is reduced by the cooling caused by the addition of hexane in the meals obtained by using solvents. This mechanism was evident in the experiments of the Doctoral Thesis of Sánchez (1989) conducted with sunflower meal; the meals obtained by using solvents had protein degradability values of 80% (continuous solvent treatment), but degradability values dropped to about 50% for meals which have been treated with the solvents after being stored in large piles (discontinuous solvent treatment).

Different chemicals have been used to protect the protein against ruminal degradation, such as acids, aldehydes and tannins, among others. Because the effects of malic acid as a protective agent against protein degradation in the rumen were investigated in this Thesis, this section will only deal with the use of organic acids. The characteristics of the main organic acids that are used in animal feeding are presented in Table 1.

Table 1. Major organic acids used as feed additives and chemical characterization.

Acid	Formula	Pk _a	Water solubility	GE (Kcal/kg)
Formic	CH ₂ O ₂	3.75	Excellent	1.386
Acetic	C ₂ H ₄ O ₂	4.75	Excellent	3.537
Propionic	C ₃ H ₆ O ₂	4.87	Excellent	4.971
Lactic	C ₃ H ₆ O ₃	3.08	Normal	3.609
Fumaric	C ₄ H ₄ O ₄	3.0 – 4.4	Regular	2.748
Malic	C ₄ H ₆ O ₅	3.4 – 5.1	Normal	2.390
Tartaric	C ₄ H ₆ O ₆	3.0 – 4.4	Normal	1.864

Source: Nielsen (2001)

Organic acids are often used as feed additives in practical feeding, but some of them have also been used at experimental level for protein protection. These acids can have a beneficial effect on digestive microbiota due to their antimicrobial effects, and are safe for the animal and the consumer as they do not produce dangerous residues. Most of the studies carried out with organic acids in ruminants have focused in fumaric and malic acids. These two organic acids are naturally occurring in biological tissues and are intermediates in the succinic cycle which transforms pyruvate into propionate (Caja *et al.*, 2003; Figure 4). Therefore, the use of malic and fumaric acid as feed additives in ruminants usually results in increased propionate production (Carro and Ungerfeld, 2015). Another effect of the addition of malic acid or its salts is a reduction of CH₄ production, due to the incorporation of hydrogen in the reduction of fumarate to succinate, thereby reducing the amount of hydrogen available in the rumen to form CH₄ (Carro and Ranilla, 2003a,b; Mohammed *et al.*, 2004; Foley *et al.*, 2007; Carro and Ungerfeld, 2015).

Figure 4. Scheme of the production of organic acids in the rumen and their final products (taken from Carro and Ungerfeld, 2015)

Although organic acids can be used as feed additives in ruminants, their handling is problematic because they are corrosive. An alternative is the use of salts (especially sodium salts), which are usually more expensive than free acids, but have the advantages of being nonabrasive and safer to manipulate (Carro and Ungerfeld, 2015). The addition of malic and fumaric acid can stabilize the ruminal pH through a stimulation of lactate utilization. *S. ruminantium* is an important lactate utilizer in the rumen and its growth and utilization of lactate was strongly stimulated by fumarate and malate (Asanuma *et al.*, 1999; Carro and Ungerfeld, 2015).

Many studies have evaluated the effects of malic acid and its salts (sodium or calcium malate) on ruminal fermentation, but the results have been highly variable. Several studies (Martin and Streeter, 1995; Carro and Ranilla, 2003a; Gómez *et al.*, 2004; Tejido *et al.*, 2005; Liu *et al.*, 2009) have reported that malate (malic acid or its salts) result in increases in the production of VFA, propionate and butyrate, as well as a reduction the acetate/propionate ratio (Tables 2 and 3, respectively). The effects of malate on CH₄ production are controversial. Malate can act as an alternative electron receptor to decrease CH₄ production while increasing propionate. Jalč and Čerešňáková (2002) observed that adding 8 mM of malic acid to rapeseed, sunflower and flax seeds incubated *in vitro* increased the propionate production by 5.8% and reduced the concentration of CH₄ by 20%. However, no reduction in CH₄ production has been reported in other studies (Carro and Ungerfeld, 2015). Carro and Ranilla (2003a) showed that the effects of malate salts on *in vitro* ruminal fermentation were influenced by the type of cereal grain in the incubation substrate. The addition of malate to cereal grains (corn, barley, wheat and sorghum) increased propionate production, total VFA and final pH for all cereals and the production of CH₄ was decreased for barley, wheat and sorghum, but no change in CH₄ production was detected for corn.

There are inconsistencies in the literature regarding the effects of malate supplementation on feed intake in ruminants, but some of these inconsistent effects seem to be due to differences in rate of supplementation (Carro and Ungerfeld, 2015). Kung *et al.* (1982) observed that supplementing malic acid (100 y 200 mg/kg body weight) to calves did not affect the DM intake, protein digestibility or nitrogen retention, and higher doses of malic acid (70, 105 and 140 g/d) neither affect DM intake in dairy cows. Castillo *et al.* (2007) indicate that the supplementation of malate to fattening heifers has beneficial effects, although the degree of response varied depending on the productive phase and the chemical form (free acid or salt). The reasons for the discrepancies among studies are unknown, but dose of malate may not be the only factor involved, and other factors as the characteristics of the diet, the chemical form of malate, and the basal content of malate in the diets fed in the different experiments can help to explain the observed differences (Carro and Ungerfeld, 2015).

Table 2. Effects of supplementation with malic acid on ruminal fermentation

References	Concentration	Effects of Malic acid							
		pH	Total VFA	Acetate	Propionate	Butyrate	Acetate:propionate	CH ₄	NH ₃ -N
Kung <i>et al.</i> (1982)	0, 70, 105, 140 g/d	NE	↑	↑	↑	↑	↓	—	NE
Montaño <i>et al.</i> (1999)	0, 80 g/d	↓	NE	↑	↓	NE	↑	↑	—
Martin <i>et al.</i> (1999)	0, 4, 8, 12 (mM)	↑	↓	↑	↓	↑	↑	—	—
Mohammed <i>et al.</i> (2004)	0, 5, 10, 20 (mM)	↑	↑	NE	↑	↑	↓	↓	NE
Ouarti <i>et al.</i> (2006)	0, 1.604, 1.112 g/mL	↓	↓	↓	↑	↑	↓	—	↓
Foley <i>et al.</i> (2009)	0, 2.5, 5.0, 7.5 (mM)	↑	↓	↓	↑	↓	↓	↓	↑
Liu <i>et al.</i> (2009)	0, 7.8, 15.6, 23.4 g/kg DM	↓	↑	↓	↑	↓	↓	—	↓
Li <i>et al.</i> (2012)	0, 5, 10 (mM)	↓	↑	↑	↑	NE	↓	↓	↓
Carrasco <i>et al.</i> (2012)	0, 1.08g/kg	NE	NE	NE	↑	NE	↓	—	↑

VFA: volatile fatty acids; CH₄: methane; NH₃-N, ammonia-nitrogen; NE: No effect of acid malic; ↑ : increase; ↓ :reduction

Table 3. Effects of supplementation with disodium malate on ruminal fermentation

References	Concentration	Effects of disodium malate							
		pH	Total VFA	Acetate	Propionate	Butyrate	Acetate:propionate	CH ₄	NH ₃ -N
Martin and Streeter (1995)* †	0, 4, 8, 12 (mM)	↑	↑	↓	↑	NE	↓	↑	↑
Martin and Streeter (1995)* †	0, 4, 8, 12 (mM)	↑	NE	↑	↑	NE	↓	↓	↑
Martin and Streeter (1995)* †	0, 4, 8, 12 (mM)	↑	↑	↑	↑	↑	↓	↓	↑
Callaway and Martin (1996) †	0, 4, 8, 12 (mM)	↑	↑	↑	↑	↑	↓	NE	↑
Martin <i>et al.</i> (1999)	0, 4, 8, 12 (mM)	↑	↓	↑	↓	↑	↑	—	—
Lila <i>et al.</i> (2004)	0, 1.24, 2.5, 5, 7.5 (mM)	NE	↑	↓	↑	↑	↓	↓	↓
Tejido <i>et al.</i> (2005)*†	0, 4, 8 (mM)	↑	↑	↑	↑	NE	↓	NE	NE

VFA: volatile fatty acids; CH₄: methane; NH₃-N, ammonia-nitrogen; NE: No effect of acid malic; ↑ : increase; ↓ : reduction

* Different feeds

† *in vitro* studies

The combination of physical and chemical treatments can be a very effective means of protecting the protein against ruminal degradation. The protection treatment should be effective and allow releasing more AA available for the host animal at intestinal level, but microbial synthesis should not be reduced. However, overheating or the use of an excessive chemical treatment might have a detrimental effect on rumen degradation and also on intestinal digestibility, thus reducing the supply of AA to the host animal. The treatment of feeds with acid solutions, with or without heat treatment, decreases the protein degradation in the rumen, increasing the feed protein flowing to the small intestine (Atwal *et al.*, 1974; Waltz and Loerch, 1986). Ouarti *et al.* (2006) showed that the combination of heat treatment with acids can act synergistically to reach higher levels of protection, reducing the disadvantages of each method separately. The combination of heat and acid treatments also presents economic advantages, due to the reduced cost of the heat treatment, the lower dose of acid required, and the reduced possibility of generating irreversible Maillard reactions which are associated with the overprotection of proteins. Protection levels depend not only on the type of acid used, but also on the dose, dilution and on the drying temperature.

The use of a lower amount of acid reduces its impact on microbial activity through the smaller effects on the ruminal environment. Wright *et al.* (2005) found no changes in the ruminal degradation of protein when rapeseed meal was treated with heat at 100°C for 120 minutes, but when the heat treatment was combined with the addition of 5% lignosulfonate the protein degradation was drastically reduced from 71.5 to 29.9%. On the contrary, Ouarti *et al.* (2006) observed no effects of a combined treatment (malic acid and heat) on ruminal degradability and intestinal digestibility of soybean meal, although the lack of effect was attributed to the low degradability of the soybean meal used in this study. Arroyo *et al.* (2011) observed that the treatment of sunflower meal with malic acid at different concentrations and temperatures increased markedly the content of rumen undegradable

protein compared with the untreated sunflower meal (49.4 vs. 23.7%; average values for all treatments). Similarly, Arroyo *et al.* (2013) treated a sunflower meal with 2N solutions of malic or orthophosphoric acid combined with a subsequent heating at 150°C for 6 h and observed that the content in rumen undegradable protein was increased by 267% regardless of the acid used; as a result, the effective intestinal digestibility of the protein was increased by 20 and 11.8% for malic and orthophosphoric acid, respectively. Díaz-Royon *et al.* (2015) suggested that combining acids (malic or orthophosphoric) and heat to treat sunflower meal and peas reduced their ruminal degradation, shifting the digestion place to the small intestine. In agreement with the results of Arroyo *et al.* (2013), Díaz-Royon *et al.* (2015) observed higher effects with malic acid than with orthophosphoric acid. In these studies, reducing the moisture and the heat applied reduced the efficacy of the treatment. However, as already pointed out these treatments can decrease the microbial protein synthesis as a result of reduced ruminal fermentation of organic matter. Despite this disadvantage, the treatments applied to sunflower meal by Arroyo *et al.* (2013) and Díaz-Royon *et al.* (2015) have clear positive effects. Thus, combined heat-acid treatments are highly effective for protecting protein sunflower meal of ruminal degradation. In addition, the malic acid added in the treatment can also have a beneficial effect on rumen fermentation, but this aspect has not yet been investigated.

1.7. OBJECTIVES

The objective of this Doctoral Thesis was to investigate some aspects of the protein degradation in the rumen, as the degradation of nitrogenous compounds associated with the cell wall of feeds, and the contribution of protein fermentation to CH₄ production in the rumen. These two general objectives were divided in the following specific objectives:

- * To determine the rumen degradation of nitrogenous fractions of the cell walls of different feeds and to assess the impact of correcting for the contribution of the solid-associated bacteria
- * To investigate the contribution of protein fermentation to CH₄ production in the rumen and to analyse the possible interactions with the type of diet.
- * To assess the efficacy of combined malic acid and heat treatments for protecting the protein against the ruminal degradation and reducing the CH₄ production *in vitro*.
- * To analyse the influence of the diet of donor' animals on the efficacy of a combined malic acid and heat treatments for protecting the protein against the ruminal degradation *in vitro*.

CHAPTER 2.-

RUMINAL DEGRADATION OF CELL WALL ASSOCIATED NITROGENOUS COMPOUNDS OF SEVERAL ¹⁵N-LABELLED FEEDS

Jorge L. Vanegas, José M. Arroyo and Javier González

*Departamento de Producción Agraria, Escuela Técnica Superior de Ingenieros
Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid,
Spain*

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2.1. INTRODUCTION

To know the digestive use of the different feed nitrogenous compounds is of great importance in the accuracy of the rationing systems in ruminants. However, most respective studies have been based on total-N measures, whereas studies performed for cell wall-bound N, usually grouped in the concepts of insoluble N in neutral detergent (NDIN) and acid detergent (ADIN) fibre solutions, are scarce. Based on the integration of N in the fibre of these two fractions, *in situ* estimates of ruminal degradation are of interest to establish its contribution to ruminal degraded N, as well as its possible effects on the intestinal digestibility of the post-ruminal N flow. Regrettably, these estimates are biased by the contamination with adherent microorganisms during feed particle residence time in the rumen-reticulum because procedures to isolate neutral detergent (NDF) and acid detergent (ADF) fibres do not allow the total detachment of ruminal adherent microorganisms. This may lead to under-estimating the ruminal degradation of cell wall-bound N, especially for NDIN (Arroyo and Gonzalez, 2013; Guevara-González *et al.*, 2015).

Isotopic markers such as ^{15}N have been used in *in situ* studies to solve the problems associated with this contamination by using two possible techniques: the rumen infusion of ^{15}N to a steady-state situation with the isolation of a sample of marked microorganisms (usually solid adherent bacteria, SAB) (Rodríguez and González, 2006) or the ^{15}N dilution technique using ^{15}N -enriched feedstuffs (Varvikko and Lindberg, 1985). Both techniques are subject to limitations and criticisms: for the first one, the possible lack of representability of the SAB sample for microorganisms adhered to specific feed particles and, for the second one, the incorporation to SAB of ^{15}N resulting from the colonised ^{15}N -enriched feed particles. However, this incorporation could be estimated. Thus, based on the relationship between the ^{15}N enrichment in SAB and in liquid associated bacteria obtained in five different experiments using intra-ruminal ^{15}N infusions, González *et al.* (2012) have shown that the

total-N supply of SAB from the colonised particles is a fixed value of 21.2%. In the above article, a similar value (19.6%) is cited as the mean of literature data from experiments carried out *in vitro* or *in vivo* in sheep, steers and cows. Therefore, a value of about 20% may be retained for this contribution. Taking into account the potential differences between the N-compounds included in NDIN and ADIN, it is interesting to compare their ruminal degradation, as well as to study their contribution to the degradation of the total N of the feed. The aim of this study was to examine the ruminal degradation of NDIN and ADIN in different vegetable feeds as well as to compare the results of the ^{15}N dilution technique using uncorrected (as is usually the case) or corrected values for microbial contamination, applying the above indicated SAB contribution.

2.2. EXPERIMENTAL

2.2.1. Tested feeds, animals and feeding

This study was performed on samples of sunflower seed (SS, oil seed type, hybrid PR63A76; Pionner), spring wheat soft grain (WG; Gazul variety; Limagrain Iberica), and wheat straw (WS) from the same crop. The wheat crop was fertilised with ^{15}N during cultivation to increase its ^{15}N abundance. Sunflower crop was not fertilised with ^{15}N but cultivated just after another ^{15}N -enriched crop, which assured sufficient ^{15}N enrichment. These samples were ground to pass a 2 mm screen for *in situ* studies and to pass a 1 mm screen for chemical composition analyses (Table 4). These three feeds were selected because they show a large range for all NDF, ADF, NDIN and ADIN fractions. Three Talaverana sheep (60 kg of average body weight, BW) equipped with ruminal cannulae (internal diameter 60 mm) were used to perform *in situ* ruminal incubations. Sheep were fed a mixed diet of lucerne hay and concentrate (in relation 2:1 on fresh matter) containing [per kg of dry matter (DM)] 165 g CP and 425 g NDF. This diet was offered at a rate of intake of 40 g DM/kg BW^{0.75} starting 8 days before beginning the incubations, in six meals per day (every 4

h, starting at 09.00 hours) with the help of automatic food dispensers. All experimental procedures were approved by the Animal Ethics Committee of Universidad Politécnica de Madrid and were in compliance with the Spanish guidelines for the care and use of animals in research, as published in the Spanish Royal Decree 53/2013 (BOE, 53/2013).

Table 4. Chemical composition (g kg^{-1} of dry matter unless otherwise indicated) of tested feeds.

Item	Sunflower seed	Wheat grain	Wheat Straw
Dry matter (g kg^{-1} of feed)	937	897	952
Ash	30.5	18.5	193
Lipids	371	4.67	1.79
Crude protein	164	153	27.0
Neutral detergent fibre	349	141	713
Acid detergent fibre	218	30.3	404
Acid detergent lignin	76.3	1.54	64.2
NDIN $\times 6.25$	19.5	23.2	9.53
ADIN $\times 6.25$	7.78	0.784	3.08
NDIN and ADIN, neutral and acid detergent insoluble N, respectively			

2.2.2. Experimental procedures

Ruminal incubations were performed using heat-sealing nylon bags (110×70 mm internal dimensions and $46 \mu\text{m}$ pore size; Saatilon, SAATI Serigrafia Iberica S.A., Almazora, Castellón, Spain) filled with approximately 3 g of feed sample (air dry basis). A total of eight bags (16 in the case of WG to obtain enough quantity of residue; 2 and 4 replicates per h, respectively) of each tested feed were incubated in the rumen of each sheep in two series of incubations for a period of 4, 24, 48 and 72 h. After being collected from the rumen, bags

were washed with tap water and stored at -20°C . After thawing, bags were washed three times for 5 minutes in a mini-turbine washing machine (Jata 580; JATA, Abadiano, Bizkaia, Spain). Bags were frozen at -20°C again and the process of thawing and washing was repeated in order to minimise contamination with adherent microorganisms. This same washing procedure was applied to three bags of each feed to obtain the 0 h value. Then, bags were oven-dried for 48 h at 60°C and weighed to determine DM disappearance. Finally, the residues of both incubations were pooled by sheep and incubation time prior analyses for total-N, NDF, ADF, NDIN, and ADIN as well as for the ^{15}N abundance in the above cited N fractions. Disappearance data of all these fractions were fitted for each animal with the exponential model of Ørskov and McDonald (1979) and effective degradability values were estimated in accordance with these authors, assuming a rumen outflow rate of 0.05 h^{-1} . Corrected degradation kinetics of N, NDIN and ADIN were established with the same model after correcting disappearance values by the microbial contamination of the respective *in situ* incubated residues. The proportions of microbial N in total-N, NDIN and ADIN were determined from their ^{15}N abundance values and those of SAB, as described in Rodríguez and González (2006): $\text{microbial N} = (\text{N}^{15}\text{ in residue} - \text{N}^{15}\text{ in feed}) / (\text{N}^{15}\text{ in SAB} - \text{N}^{15}\text{ in feed})$.

The ^{15}N abundance used as reference for tested feeds was determined on the zero incubation fractions because only the insoluble material is subject to microbial contamination. When the ^{15}N dilution technique is used in *in situ* studies SAB cannot be isolated from the marked incubated residues due to the small quantity of sample. For this reason the ^{15}N abundance of SAB was established considering that a part of their N content is derived from the ^{15}N -labelled feed particles. As previously indicated by González *et al.* (2012) this part was assumed as 20% of the total-N content of SAB. Therefore, the ^{15}N abundance in SAB considered for all tested fractions of each feed was estimated as the

weighted mean of the atmospheric natural abundance (80 % of 0.3663 atoms %) and 20 % of the ^{15}N abundance of the zero incubation DM residues (X) as: $0.8 \times 0.3663 + 0.2X$.

2.2.3. Chemical analyses

Chemical composition of tested feeds was determined by the methods described by the Association of Official Analytical Chemists (2000) for DM (procedure 934.01), ash (procedure 967.05), ether extract (procedure 920.39) and CP ($\text{N} \times 6.25$; procedure 968.06). An Ankom fibre analysis system (Model 220; Ankom Technology Corp., Macedon, NY, USA) was used to determine NDF (Van Soest *et al.*, 1991), sequential ADF and acid detergent lignin (ADL) (Robertson and Van Soest, 1981) in samples and incubated residues. Analyses of NDF were performed with α -amylase but without sodium sulfite. The NDF and ADF were expressed inclusive of residual ash. Fibre analyses of SS were performed on samples defatted with the previously indicated ether extract procedure. The NDIN and ADIN were determined in the NDF and ADF residues, respectively. Nitrogen and ^{15}N abundance were determined simultaneously by combustion in an EA Carlo Erba 1108 coupled in continuous flow to an Isotope Ratio Mass Spectrometer model Micromass Isochrom (Cheshire, UK)

2.2.4. Statistical methods

Degradation kinetics were fitted using non-linear regression models. Degradation parameters were compared by analysis of variance considering the tested effect (x ; comparisons among fractions or corrected vs. non-corrected values) and sheep (s) in the model ($y_{ij} = \mu + x_i + s_j + \varepsilon_{ij}$). Comparisons of means among fractions were performed using the Tukey–Kramer test. Effects of the factors were declared significant at $P \leq 0.05$ and trends were discussed at $P > 0.05$ and $P \leq 0.1$. All statistical analyses were performed using the SAS software (SAS, 2008).

2.3. RESULTS

The ^{15}N abundance in tested feeds and in their insoluble fractions (0 h samples) is shown in Table 5. Values of both sample types were close in SS but reached some important difference in WG and WS. Thus, on average, regardless of N fractions 0 h values were 3.60% (WG) and 3.93% (WS) higher than in the whole feeds. Values of ^{15}N abundance in total-N and NDIN were always close, whereas they decreased in ADIN. On average, when compared with total-N and NDIN, these reductions were small in SS (1.28%) and large in WG and WS (9.95%, and 9.24%, respectively).

Table 5. Abundance of ^{15}N (atoms%) in whole feeds, their insoluble fraction (0 h sample) and their respective N-bounded to neutral detergent (NDIN) and acid detergent (ADIN) fibre fractions.

Sample	Total-N	NDIN	ADIN
Sunflower seed			
Whole	0.4065	0.4105	0.4004
0 h sample	0.4020	0.4060	0.4017
Wheat grain			
Whole	0.6916	0.6973	0.6166
0 h simple	0.7146	0.7109	0.6506
Wheat Straw			
Whole	0.6041	0.5877	0.5432
0 h sample	0.6211	0.6209	0.5612

Contamination with microbial N of total-N, NDIN and ADIN through incubation times is shown in Fig. 5. Microbial contamination increased with the rumen residence time in all tested fractions except for a decrease in ADIN values between times 4 and 24 h in SS and

WG. Also, contamination decreased in the following order: total-N, NDIN, and ADIN, except in the above cited 4-h samples which provided the opposite pattern.

Microbial non-corrected values of ruminal degradation parameters of DM and fibre fractions are shown in Table 6. The soluble fraction (a) was higher for DM than for fibre fractions in all feeds and it was also higher for NDF than for ADF in SS. The opposite behaviour was shown for the undegradable fraction (r), although in WS no difference was recorded. Values of k_d in WG were higher for the feed-DM than for the fibre fractions, with no differences between NDF and ADF, where as in WS differences were not recorded. Finally, in SS this rate was higher in NDF than in ADF whereas the value for the feed-DM was intermediate and not different from these two fractions. Results show reduced ED estimates for fibre fractions compared with the whole feeds as well as for ADF versus NDF in concentrates (Table 6).

Figure 5. Contamination with microbial N of feed residues (black bars) and their N-bound to neutral detergent (NDIN; grey bars) or acid detergent (ADIN; white bars) fibre fractions. (A) sunflower seed, (B) wheat grain and (C) wheat straw. Bars are SD.

Table 6. Kinetic degradation parameters of dry matter (DM) and neutral (NDF) and acid (ADF) detergent fibres of tested feeds

Items ^a	Feed fractions			
	DM	NDF	ADF	SEM ^b
Sunflower seed				
<i>a</i>	0.509 ^c	0.067 ^d	0.000 ^e	0.003
<i>b</i>	0.209 ^d	0.240 ^c	0.161 ^e	0.0025
<i>r</i>	0.283 ^e	0.693 ^d	0.839 ^c	0.0023
k_d (h ⁻¹)	0.169 ^{cd}	0.254 ^c	0.126 ^d	0.0254
ED	0.666 ^c	0.264 ^d	0.112 ^e	0.0029
Wheat grain				
<i>a</i>	0.283 ^c	0.002 ^d	0.000 ^d	0.0004
<i>b</i>	0.670 ^d	0.721 ^c	0.542 ^e	0.0076
<i>r</i>	0.047 ^e	0.277 ^d	0.458 ^c	0.0077
k_d (h ⁻¹)	0.513 ^c	0.216 ^d	0.236 ^d	0.0126
ED	0.893 ^c	0.584 ^d	0.447 ^e	0.0094
Wheat straw				
<i>a</i>	0.177 ^c	0.084 ^d	0.088 ^d	0.0012
<i>b</i>	0.283	0.339	0.367	0.0236
<i>r</i>	0.540	0.577	0.545	0.0225
k_d (h ⁻¹)	0.0452	0.0327	0.0283	0.00416
ED	0.304 ^c	0.210 ^d	0.210 ^d	0.0036

^a *a*, *b* and *r*, soluble, non-soluble degradable and undegradable fractions, respectively. k_d : fractional degradation rate of *b* fraction. ED, effective degradability; ^b SEM, standard error of the mean; *df* =4; ^c, ^d, ^e Values with different superscripts are different at $P < 0.05$.

The evolution of non-corrected disappearance values of all N-tested fractions in WS as well as those of ADIN in WG did not fit with the proposed model. The evolution in each sheep of non-corrected and corrected disappearances in these cited cases are shown in Figure

6, in which it can be observed that effects of microbial contamination on disappearance measures were large among sheep and that microbial correction reduced this variability.

Values of ruminal degradation parameters for total-N, NDIN and ADIN non-corrected and corrected for microbial contamination are shown in Table 7. The microbial contamination correction led in SS to increased ED values for total-N, NDIN and ADIN. In WG these increases were recorded as a trend for both total-N ($P = 0.064$) and NDIN ($P = 0.096$). In SS these augmentations were mainly due to the increment of the b fraction and also of the k_d rate for NDIN and ADIN. A similar behaviour was recorded in WG, although for NDIN the increase of b was only numerical.

In corrected values, large reductions of fraction a were shown between total-N and N of fibre fractions in all feeds, whereas the opposite effect was observed for the r fraction in SS. A similar trend was observed in WG and WS but no differences were shown between NDIN and total N r values in spite of the large differences recorded between both values. Variations in the b fraction were conditioned by those occurred in the above cited parameters. Thus, the b value for total-N was lower than for NDIN and ADIN in SS and WS whereas in WG it was higher than for ADIN. In all feeds the a fraction was higher in NDIN than in ADIN and the opposite variation was shown for the r fraction although in SS and WS this difference was only numerical. The b fraction was also higher for NDIN, although only significantly in WG. No differences for k_d were shown among the three tested fractions in SS, whereas this rate was higher for total-N than for N of fibre fractions in WG and for ADIN than for total-N and NDIN in WS. Results also show greater ED estimates for total-N than for N of fibre fractions in all feeds (although in WG the difference with NDIN was not significant) and for NDIN than for ADIN in WG.

Figure 6. Evolution of microbial non-corrected (open symbols) and corrected (closed symbols) values of N disappearance when exponential fitting of non-corrected values was originally not possible. (A) insoluble N in acid detergent fibre (ADIN) of wheat grain. (B, C and D) Total-N, insoluble N in neutral detergent fibre (NDIN) and ADIN of wheat straw, respectively. The different symbols and lines correspond to three sheep.

Table 7. Differences in ruminal degradation parameters between values uncorrected (U) and corrected (C) by microbial contamination of total-N and N-bound to neutral (NDIN) or acid (ADIN) detergent fibres as well as between corrected values of these three fractions

Items ^a	Total N				NDIN				ADIN				SEM ^c	P
	U	C	SEM ^b	P	U	C	SEM ^b	P	U	C	SEM ^b	P		
Sunflower seed														
a	0.717	0.717 ^d	0.0001	0.183	0.057	0.069 ^e	0.0058	0.291	0.039	0.044 ^f	0.0026	0.340	0.0002	<0.001
b	0.235	0.253 ^e	0.0019	0.022	0.476	0.606 ^d	0.0277	0.079	0.439	0.562 ^d	0.0161	0.033	0.0176	<0.001
r	0.049	0.031 ^e	0.0019	0.023	0.467	0.325 ^d	0.0306	0.082	0.521	0.394 ^d	0.0150	0.027	0.0176	<0.001
k _d (h ⁻¹)	0.197	0.197	0.0016	0.655	0.140	0.233	0.0176	0.065	0.128	0.279	0.0134	0.015	0.0298	0.261
ED	0.903	0.917 ^d	0.0011	0.013	0.402	0.559 ^e	0.0239	0.043	0.347	0.520 ^e	0.0064	0.003	0.0197	<0.001
Wheat grain														
a	0.254	0.254 ^d	0.0001	0.918	0.090	0.090 ^e	0.0001	0.423	-	0.014 ^f	-	-	0.0002	<0.001
b	0.726	0.736 ^d	0.0024	0.092	0.829	0.865 ^d	0.0097	0.117	-	0.544 ^e	-	-	0.0276	0.003
r	0.020	0.010 ^e	0.0024	0.092	0.082	0.045 ^e	0.0097	0.116	-	0.442 ^d	-	-	0.0278	<0.001
k _d (h ⁻¹)	0.465	0.461 ^d	0.0089	0.745	0.286	0.301 ^e	0.0011	0.011	-	0.321 ^e	-	-	0.0245	0.019
ED	0.908	0.917 ^d	0.0018	0.064	0.793	0.830 ^d	0.0087	0.096	-	0.482 ^e	-	-	0.0247	<0.001
Wheat straw														
a	-	0.436 ^d	-	-	-	0.086 ^e	-	-	-	0.000 ^f	-	-	0.0103	<0.001
b	-	0.348 ^e	-	-	-	0.552 ^d	-	-	-	0.481 ^d	-	-	0.0264	0.013
r	-	0.216 ^e	-	-	-	0.362 ^{de}	-	-	-	0.519 ^d	-	-	0.0353	0.010
kd (h-1)	-	0.116 ^e	-	-	-	0.063 ^e	-	-	-	0.417 ^d	-	-	0.0444	0.010
ED	-	0.607 ^d	-	-	-	0.386 ^e	-	-	-	0.426 ^e	-	-	0.0224	0.002

^a *a*, *b* and *r*, soluble, non-soluble degradable and undegradable fractions, respectively. *k_d*, fractional degradation rate of *b* fraction. ED, effective degradability; ^b SEM, standard error of the mean between uncorrected and corrected values; *df* =2; ^c SEM and *P* values: among corrected N fractions; *df* =4; ^{d, e, f} Corrected values with different superscripts are different at *P* <0.05

2.4. DISCUSSION

2.4.1. *Microbial contamination*

Positive or negative differences of ^{15}N abundance between the whole feed and its insoluble fraction have been previously shown in a large collection of non-labelled feeds by Rodríguez and González (2006) which was explained by them by non-systematic differences in the ^{15}N fixation by the different tissues. Also, these authors showed that small differences in the ^{15}N abundance in the sample used as reference can lead to large errors in microbial contamination estimates, supporting the interest of using ^{15}N values from specific reference samples instead of those derived from the whole feed. As a consequence, values of 0 h samples were adopted as initial ^{15}N abundance because, as previously indicated, only the insoluble materials are subject to microbial contamination. The decrease of ^{15}N abundance of ADIN in all samples should be noted for its potential interest on the accuracy of this analysis. These decreases may suggest that ^{15}N does not distribute evenly in the different cell wall structures. However, considering the large magnitude of these decreases in WG and WS, a more probable cause may be the dilution of the ^{15}N content of ADIN by the absorption of N by fibre particles during the boiling in the solution of cetyl-trimethylammonium bromide. This absorption will depend on the fibre polarity, which agrees with the lower reduction shown in SS than in wheat samples. Thus, the high lignification and the lipid content of SS hulls as well as its important waxy cuticle (Jarrige, 1988) may result in a high hydrophobicity of this fibre, limiting the indicated N absorption. The hypothesis of N-absorption, if true, implies errors in ADIN determinations and, therefore, further studies are needed to clarify this point as ADIN is a nutritional parameter commonly used.

The maximum microbial contamination of total-N reached in WS (70.0%) was somewhat lower than that reported for a barley straw (80.9%) (Rodríguez and González, 2006). However, the contents of cellulose (measured as ADF-ADL) and lignin were,

respectively, 20% lower and 30% higher in the current sample, which may explain this difference as both of these factors affect positively for cellulose and negatively for lignin the microbial contamination (BOE, 53/2013). In addition, it should be considered that the freezing and washing cycle of bags was duplicated in the present study. Current results were also close to that of 70.9% shown by Varvikko and Lindberg (1985) for a 24 h-incubated barley straw residue and lower than that of 82% observed by Bernard *et al.* (1988) for a 48 h-incubated wheat straw. The increasing evolution of contamination over time is associated with the development of micro-colonies and agrees with previous results (Rodríguez and González, 2006; Nocek, 1988; Krawielitzki *et al.*, 2006). The decreasing contamination order shown in general for total-N, NDIN and ADIN agrees with previous results obtained in several types of digesta samples, which showed a partial and accumulative microbial decontamination with the application of NDF and sequential ADF procedures (Arroyo and González, 2013; Guevara-González *et al.*, 2015). The exception to this behaviour observed at 4 h of incubation in SS and WG may be associated with the dilution of microbial N in a still high N content of incubated residues, which decreased from total-N to ADIN.

In hypoproteic and fibrous feeds such as cereal straws, the high microbial contamination leads even to increases of the N content of residues with increasing incubation times, which prevents determining the ruminal CP degradation if this contamination is not corrected (Rodríguez and González, 2006; Varvikko and Lindberg, 1985; Tamminga, 1993). This problem is extended for the same reasons to the N bound to fibre fractions, as shown in present results in WS, as well as for ADIN of WG. The under-evaluations of the ED of total-N due to the lack of contamination correction were low in SS (1.52%) and in WG (1.02%). It was also low for the ED of NDIN in WG 4.43%) but important for both NDIN (28.0%) and ADIN (33.3%) in SS. In the remaining cases shown in Figure 6, these errors were large but not measurable, as degradation kinetics could not be fitted.

2.4.2. Feed degradation

The low a fractions estimated for NDF and ADF (in several cases close to zero) show a low content of soluble fibre in these feeds, which agrees with previous results in sunflower hulls and wheat straw (Abad *et al.*, 2013). Also, they are indicative that the losses of undegraded particles from the bag, if any, were small. The comparison of r values among the different tested fractions in SS and WG shows a limitation to fibre degradation that is higher for ADF than for NDF.

This fact should be associated with the protective effect of lignin against the progression of the microbial attack (Akin, 1979; Chesson and Forsberg, 1988). Conversely, the similar degradation kinetics in WS of both fibre fractions, which lead to the same ED estimates of NDF and ADF, support a similar degradation progression in the associated tissues, probably because they are mainly constituted by lignified fibrous materials. As similar degradation rates of NDF and ADF were also observed in WG, the same behaviour of degradation progression in the tissues associated with both fibre fractions may also be supposed. In SS the degradation rate observed in NDF is twice that of ADF and must be associated with a greater contribution of seed tegument and pericarp in these both fractions, respectively. In addition, the outer cuticle layer of pericarp hinders microbial colonization (Akin, 1979), contributing to the low ADF degradation rate. The intermediate k_d value of DM-SS between those NDF and ADF could be associated with the toxicity for microorganisms derived from the seed-fat fermentation (Palmquist and Jenkins, 1980), which should limit the degradation progression. For DM, degradation parameters and ED estimates are in acceptable agreement with previous literature data for SS (Sauvant *et al.*, 2002; Mustafa *et al.*, 2003), WG (Bacha *et al.*, 1992; Arroyo *et al.*, 2009) and WS (Chaudhry, 2000; Shawrang *et al.*, 2013). Present ED estimates for NDF in the latter feed also agree with those indicated by these authors (Chaudhry, 2000; Shawrang *et al.*, 2013).

The high ED of total-N in SS supports previous values from Mustafa *et al.* (2003) or that assumed by Sauvant *et al.* (2002). On the other hand, its cell wall-bound N had a lower ED (0.559 for NDIN and 0.520 for ADIN) due to the large increase (10.7 and 12.9 times for NDIN and ADIN, respectively) of the proportion of undegradable contents. The similar ED value of these two N-fractions contrasts with the large differences observed for the ED of NDF and ADF, showing that both N fractions are less affected by the degradation inhibitory factors associated with the structure and lignification of the cell wall. The high ED of total-N estimated in WG agrees with data recorded in different samples cultivated in Spain. Thus, in four samples Bacha *et al.* (1992) showed a mean non-corrected microbial value of 0.914. The NDIN fraction of WG showed high availability in the rumen (0.830). This availability is largely lower for the ADIN (0.482), mainly as a consequence of the nearly 10-fold increase in the proportion of undegradable content (*r*) in relation with NDIN. However, this fact has little practical importance due to the very low ADIN content of this grain. The microbial corrected ED of total-N in WS (0.670) was close to those obtained for this by-product by Bernard *et al.* (1988) and Ould-Bah *et al.* (1988) 0.710 and 0.647, respectively. Rodríguez and González (2006) showed a corrected value of 0.770 for a barley straw of a better quality than the straw used in this study; thus it had 44.7 g CP kg⁻¹ of DM and showed lower proportions of NDIN (27.4% vs. 35.6% of total-N) and ADIN (6.24% vs. 11.5% of total-N). In agreement with the close ED values observed in WS for NDF and ADF, results for NDIN and ADIN were also similar, attaining about 60% on average than the ED observed for total-N. The similarity between ED of NDIN and ADIN is a consequence of the offset between the smaller proportion of degradable compounds of ADIN and its higher degradation rate compared with NDIN. A faster initial degradation in ADIN than in NDIN and similar microbial corrected ED values of both N-fractions have also been observed in an Italian ryegrass hay of poor quality (Arroyo and González, 2013).

2.5. CONCLUSIONS

Results show that procedures of NDF and ADF isolation do not remove a large fraction of microorganisms adhered during rumen incubation and therefore large under-evaluations of ED of NDIN and ADIN may occur when this contamination is not considered. Ruminal degraded NDIN reached a considerable importance contributing in 7.25% (SS), 13.7% (WG), and 20.3% (WS) to the degraded N. In highly lignified fibrous feeds such as SS and WS, ADIN degradation is similar to that of NDIN, whereas in WG it was largely more limited. Contributions of ADIN degradation to total degraded N were 2.69%, 0.27% and 7.25% in SS, WG and WS, respectively.

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CHAPTER 3.-

INFLUENCE OF PROTEIN FERMENTATION AND CARBOHYDRATE SOURCE ON *IN VITRO* METHANE PRODUCTION

Jorge Leonardo Vanegas, Javier González, María Dolores Carro

*Departamento de Producción Agraria, Escuela Técnica Superior de Ingenieros
Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid,
Spain*

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3.1. INTRODUCTION

Reducing methane (CH₄) emissions without negatively affecting animals' productivity is currently an important goal of ruminants' nutritionists, as CH₄ represents a significant loss of energy for the host animal and contributes to global warming (Moss *et al.*, 2000). Several factors have been shown to influence enteric production of CH₄, but dietary characteristics are recognized as one of the most important (Kumar *et al.*, 2014). Many studies have investigated the influence of the amount and type of carbohydrate in the diet, and it is well known that the fermentation of diets rich in structural carbohydrates yields higher CH₄ emissions compared with that of diets high in soluble carbohydrates (Johnson and Johnson, 1995). In contrast, to our knowledge no work has been conducted to specifically assess the contribution of protein fermentation to ruminal CH₄ production, although protein degradation *in vitro* has been shown to generate lower amounts of gas than carbohydrates' degradation (Cone and Van Gelder, 1999; Jentsch *et al.*, 2007). The process of degradation of dietary protein and assimilation into microbial protein can result in either a net consumption or a net production of hydrogen, as the protein synthesis utilizes reducing equivalents and the synthesis of amino acids can result in either production or utilization of reducing equivalents (Knapp *et al.*, 2014), but there is a lack of information on how these processes may affect CH₄ production.

Several studies have shown that providing protein-N in addition to NH₃-N stimulated the growth of rumen bacteria and enhanced fibre digestion (Molina-Alcaide *et al.*, 1996; Griswold *et al.*, 1996), but to our knowledge no study has specifically addressed the influence of the substrate characteristics on the response to protein-N sources. Some authors (Cruz Soto *et al.*, 1994) have hypothesized that the degree of stimulation by protein-N is related to carbohydrate fermentation rate, with greater responses occurring with more rapidly fermented substrates, but others have reported a marked stimulation of microbial growth with fibrous

slowly-fermented substrates (Carro and Miller, 1999; Ranilla *et al.*, 2001). To our knowledge no study has specifically addressed the influence of the substrate characteristics (carbohydrate and N-sources) on CH₄ production. Our hypothesis was that the contribution of protein fermentation to CH₄ emission might vary with the type of carbohydrate available to ruminal microbes. The objective of this experiment was therefore to investigate the contribution of protein fermentation to *in vitro* CH₄ production and to assess its influence on the fermentation of two pure substrates composed by carbohydrates of variable fermentation rate.

3.2. MATERIALS AND METHODS

3.2.1. Animals and feeding

Four adult rumen-fistulated sheep (65.2 ± 2.15 kg body weight) were used as rumen fluid donors for the *in vitro* incubations. Animals were placed in individual pens with free access to water and a mineral-vitamin mixture. Sheep were fed a 2:1 mixed diet of lucerne hay and a commercial concentrate at energy maintenance level (44 g of dry matter (DM)/kg body weight^{0.75}; Agricultural Research Council, 1984) distributed in two equal meals. The diet contained 913, 168, 426 and 269 g of organic matter, crude protein, neutral detergent fibre (NDF) and acid detergent fibre (ADF) per kg of dry matter, respectively. Animals' management and rumen content withdrawal were 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) in line with the European Directive for the Protection of animals used for scientific purposes (Directive 2010/63/UE). All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Polytechnic University of Madrid.

3.2.2. *Substrates, N sources and in vitro incubations*

Two N-free substrates were prepared by mixing (DM basis) maize starch (Cerestar 3406, Manuel Riesgo S.A., Madrid, Spain) and cellulose (sodium carboxymethylcellulose, Manuel Riesgo S.A., Madrid, Spain) in proportions of 75:25 and 25:75 (STAR and CEL, respectively). Samples (160 mg of DM) of each substrate were weighed into 120-ml serum bottles. Three forms of added N were evaluated: ammonia-N (NPN; NH_4Cl), casein (CA; Manuel Riesgo S.A., Madrid, Spain) and purified soybean protein (SP; SUPRO®500E, Protein Technologies International, Corby, Northants, UK). Casein was chosen for its rapid degradation rate by ruminal microbes and SP for being more slowly degraded (Eschenlauer *et al.*, 2002; Broderick *et al.*, 2004). The lower degradation rate of SP was tested in the laboratory before conducting the experiment by incubating CA and SP as only substrates as described below. Values of gas production from SP fermentation were 0.80, 0.81, 0.84, 0.88, 0.90 and 0.96 of those for CA at 2, 4, 6, 9, 12 and 16.5 h of incubation, respectively (results now shown).

Five treatments were made by replacing NPN with either CA or SP at levels of 0 (NPN), 50 (CA50 and SP50) and 100% (CA100 and SP100) of total N. Isonitrogenous solutions of each treatment were prepared in buffer (pH=8.4) and 2 ml of the corresponding solution (containing 5.6 mg N) was added to each bottle immediately before sealing. All N treatments were applied at a level of 35 mg of N/g of substrate organic matter (OM), which is above the mean value of 32 mg of N/g OM apparently fermented in the rumen recommended by the Agricultural Research Council (1984) for optimal microbial growth. For the calculations of the amount of N to be added to each culture it was assumed that all N sources and both substrates had a potential degradability of 100%.

Rumen content from each donor sheep was obtained immediately before the afternoon feeding, strained through 4 layers of cheesecloth and mixed with a buffer solution in a 1:4

ratio (vol/vol) at 39°C under continuous flushing with CO₂. The medium of Goering and Van Soest (1970) was modified by replacing the (NH₄)HCO₃ with NaHCO₃ and excluding the trypticase, and the resulting N-free solution was used as buffer. A solution of isobutyrate, isovalerate and valerate acids were added to each mixture of rumen fluid plus buffer to achieve final concentrations of 0.3, 0.9 and 0.7 mM, respectively, in order to stimulate the growth of cellulolytic bacteria (Hume, 1970).

Bottles were sealed with rubber stoppers and aluminium caps, incubated at 39°C, and withdrawn 16.5 h after inoculation (corresponding to a mean passage rate from the rumen of 0.06 per h). Total gas production was measured using a pressure transducer (HD2304.0 pressure gauge, DELTA OHM, Italy) and a calibrated syringe. A gas sample (10 ml) was removed from each bottle and stored in a vacuum tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH₄. Bottles were then uncapped and the pH was measured immediately with a pH meter (*Crison Basic 20*, Crisson Instruments, Barcelona, Spain). Three ml of content was added to 3 ml of deproteinising solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per l) for volatile fatty acid (VFA) determination and 2 ml were added to 2 ml 0.5 M HCl for NH₃-N analysis.

Incubations were performed in four different days, and each day the rumen fluid from one sheep was used as inoculum. A total of 32 bottles were incubated each day: 20 bottles with substrate and N sources (2 for each substrate and N treatment), 10 bottles only with N sources (2 per N treatment), and 2 additional bottles with only buffered ruminal fluid from sheep (blanks).

3.2.3. Chemical analyses

Dry matter (ID 934.01), ash (ID 942.05) and N (ID 984.13) content of the diet fed donor sheep were determined according to the Association of Official Analytical Chemists (1999). The NDF and ADF analyses were carried out according to Van Soest *et al.* (1991)

using an ANKOM220 Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF and ADF, and they were expressed exclusive of residual ash.

Concentrations of $\text{NH}_3\text{-N}$ in ruminal fluid were determined using a spectrophotometer by the method of phenol-hypochlorite (Weatherburn, 1967) and those of VFA by gas chromatography as described by Carro *et al.* (1992). Analysis of CH_4 was carried out following the procedure of Martínez *et al.* (2010a) using a gas chromatograph (Shimadzu GC 14B; Shimadzu Europa GmbH, Duisburg, Germany) equipped with a flame ionization detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain).

3.2.4. Calculations and statistical analyses

The amounts of VFA produced in each culture were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. The volume of gas produced was corrected for temperature (0°C and 1 atm, respectively) before calculating the amount of CH_4 produced. The amount of organic matter apparently fermented (OMAF) in each bottle was estimated from acetate, propionate and butyrate production as described by Demeyer (1991). No correction for the values measured in the blanks was made for calculations of VFA and CH_4 . Values determined in the 2 bottles incubated for each inoculum and experimental treatment were averaged before statistical analysis (4 values per experimental treatment). Values in the 2 bottles were similar, with CV lower than 2.0%.

Data for each protein source (CA and SP) were analysed independently as a mixed model using the PROC MIXED of SAS (SAS, 2012). In the culture substrate (STAR and CEL) the effects of N source (0, 50 and 100% protein-N), substrate and N source x substrate were considered fixed, and inoculum effect was considered random. In the cultures with only the N forms added (no substrate), the effect of source (0, 50 and 100% protein-N) was

considered fixed, and inoculum effect was considered random. As the substitution of NPN by protein-N was made at equally spaced intervals (0, 50 and 100%), orthogonal polynomial contrasts were used to test for linear and quadratic effects of protein-N addition. Significance was declared at $p < 0.05$, whereas $p < 0.10$ values were considered to be a trend.

3.3. RESULTS

The effects of replacing NPN by CA and SP are shown in Tables 8 and 9, respectively. There were no N source x substrate interactions ($P = 0.114$ to 0.937) for any measured variable with the exception of CH_4 /total VFA ratio for both proteins ($P = 0.011$ and 0.009 for CA and SP, respectively), CH_4 production for SP ($p = 0.002$), and propionate production ($P = 0.046$) for CA. When these parameters were analysed independently for each substrate, the replacement of NPN by increased levels of CA or SP led to linear increases ($P = 0.023$ and 0.003 , respectively) of CH_4 /total VFA ratio with STAR, but the CH_4 /total VFA ratio tended to be decreased with CEL ($p = 0.053$ and 0.056 for CA and SP, respectively). Production of CH_4 was linearly increased ($P < 0.001$) when SP was supplied to STAR substrate, and quadratically increased ($P = 0.002$) with CEL substrate. Replacing NPN by increased levels of CA increased propionate production with CEL (quadratic, $P = 0.044$), but had no effect with STAR ($P = 0.165$). In addition, a trend to N source x substrate interaction was observed for CH_4 production with CA ($P = 0.065$) and for propionate production with SP ($P = 0.066$).

Table 8. Effects of replacing non-protein nitrogen (NPN) by casein on the in vitro fermentation of substrates composed by starch and cellulose in ratios 75:25 (STAR) or 25:75 (CEL) incubated in batch cultures of mixed ruminal micro-organisms for 16.5 h*

Item								<i>P</i> -value			
	STAR			CEL				Nitrogen source [†]		Substrate	Nitrogen source x
	NPN	CA50	CA100	NPN	CA50	CA100	SEM	L	C		
Gas (μmol)	2076	1991	2195	931	1102	1115	58.3	0.473	0.021	<0.001	0.114
CH ₄ (μmol)	259	358	400	195	245	270	13.4	<0.001	<0.001	<0.001	0.065
Volatile fatty acid (μmol)											
Total	863	955	1021	313	463	595	30.5	0.001	<0.001	<0.001	0.166
Acetate	472	567	609	179	278	355	20.8	<0.001	<0.001	<0.001	0.598
Propionate	199	173	172	66.0	76.0	97.3	10.52	0.478	0.499	<0.001	0.046
Butyrate	167	172	173	55.0	75.5	87.2	9.41	0.205	0.141	<0.001	0.398
Isobutyrate	5.38	8.65	12.9	2.83	6.13	11.0	0.959	0.004	<0.001	0.010	0.925
Isovalerate	7.08	15.6	24.5	7.20	15.1	24.8	1.321	<0.001	<0.001	0.999	0.948
Valerate	12.6	18.4	30.6	2.64	12.2	19.5	2.366	0.005	<0.001	<0.001	0.566
Acetate/Propionate (mol/mol)	2.40	3.29	3.61	2.99	3.70	3.64	0.278	0.011	0.043	0.150	0.597
CH ₄ /VFA (mol/mol)	0.300	0.375	0.391	0.623	0.529	0.454	0.0418	0.560	0.384	<0.001	0.011
Organic matter apparently fermented (mg)	82.4	87.8	91.3	29.6	41.4	50.3	3.08	0.014	0.001	<0.001	0.194
NH ₃ -N (mg/l)	295	252	246	285	253	247	13.7	0.015	0.053	0.815	0.907

* NPN, CA50 and CA100: treatments whereby ammonia-N (NPN; NH₄Cl) was replaced by casein-N at levels 0, 50 and 100% of total N, respectively.

[†] Linear (L) and quadratic (C) effects of casein.

Table 9. Effects of replacing non-protein nitrogen (NPN) by purified soybean protein on the in vitro fermentation of substrates composed by starch and cellulose in ratios 75:25 (STAR) or 25:75 (CEL) incubated in batch cultures of mixed ruminal micro-organisms for 16.5 h*

Item								<i>P</i> -value			
	STAR			CEL				Nitrogen [†]		Substrate	Nitrogen source x
	NPN	SP50	SP100	NPN	SP50	SP100	SEM	L	C		
Gas (μmol)	2076	1903	2063	931	937	1138	59.1	0.176	0.016	<0.001	0.173
CH ₄ (μmol)	259	391	390	195	210	253	13.4	<0.001	<0.001	<0.001	0.002
Volatile fatty acid (μmol)											
Total	863	923	959	313	411	497	23.8	0.005	<0.001	<0.001	0.210
Acetate	472	576	590	179	244	307	14.2	<0.001	<0.001	<0.001	0.224
Propionate	199	145	156	66.0	65.7	73.5	13.08	0.089	0.825	<0.001	0.066
Butyrate	167	169	175	55.0	82.0	86.7	6.50	0.091	0.066	<0.001	0.261
Isobutyrate	5.38	7.54	8.80	2.83	4.30	7.00	0.850	0.051	0.001	0.003	0.707
Isovalerate	7.08	11.4	13.9	7.20	9.65	15.9	0.923	0.002	<0.001	0.872	0.162
Valerate	12.6	13.2	15.6	2.64	4.57	7.52	1.539	0.572	0.033	<0.001	0.937
Acetate/Propionate (mol/mol)	2.40	4.23	4.01	2.99	3.85	4.19	0.527	0.070	0.228	0.437	0.323
CH ₄ /VFA (mol/mol)	0.300	0.424	0.407	0.623	0.511	0.509	0.0424	0.931	0.855	<0.001	0.009
Organic matter apparently fermented (mg)	82.4	85.8	87.0	29.6	37.6	45.0	2.26	0.024	0.002	<0.001	0.089
NH ₃ -N (mg/l)	295	232	175	285	223	187	13.8	<0.001	<0.001	0.847	0.699

* NPN, SP50 and SP100: treatments whereby ammonia-N (NPN; NH₄Cl) was replaced by soybean-N at levels 0, 50 and 100% of total N, respectively.

[†] Linear (L) and quadratic (C) effects of soybean protein

Final pH values ranged from 6.56 to 6.89 (values not shown) and were not affected ($P > 0.05$) by N treatments, possibly due to the high buffer capacity of the incubation medium. Replacing NPN by increasing amounts of CA augmented the amount of both gas (quadratic, $P = 0.021$) and CH_4 (linear and quadratic, $P < 0.001$). Similarly, total VFA production and OMAF were linearly ($P = 0.001$ and 0.014 , respectively) and quadratically ($P < 0.001$ for both) increased with increasing CA supplementation. There were no effects ($P > 0.05$) of CA supply on propionate and butyrate production, but acetate, isobutyrate, isovalerate and valerate productions were increased (quadratic, $P < 0.001$) and $\text{NH}_3\text{-N}$ concentrations decreased (linear, $P = 0.015$) by supplying increased amounts of CA.

Substituting of NPN by SP also increased both gas (quadratic, $P = 0.016$) and CH_4 (linear and quadratic, $P < 0.001$) production, as well as total VFA production (linear, $P < 0.05$; quadratic, $P < 0.001$) and OMAF (linear, $P = 0.024$; quadratic, $P = 0.002$). Quadratic increases ($P < 0.001$ to 0.033) in the production of acetate, isobutyrate, isovalerate and valerate, and a trend to increased butyrate production ($P = 0.066$) were observed by adding SP to the cultures. Concentrations of $\text{NH}_3\text{-N}$ were linearly and quadratically decreased ($P < 0.001$) by replacing NPN with increased amounts of SP.

As expected, there were marked differences in fermentation parameters between the two substrates. With all N sources, STAR fermentation resulted in higher ($P < 0.001$) OMAF and production of gas, CH_4 and total VFA, but lower $\text{CH}_4/\text{total VFA}$ ratios compared with fermentation of CEL. The amounts of all individual VFA produced, excepting those of isovalerate, were higher ($P < 0.010$) with STAR than with CEL. In contrast, no differences ($P > 0.05$) between substrates were observed in the acetate/propionate ratio.

The effects of replacing NPN by CA and SP in the batch cultures in the absence of substrate are shown in Tables 10 and 11, respectively. Adding both protein-N sources to the cultures resulted in increased CH_4 (linear, $P = 0.008$ and 0.019 for CA and SP, respectively;

quadratic, $P \leq 0.001$ for both proteins) and total VFA production (linear, $P = 0.008$ and <0.001 for CA and SP, respectively; quadratic, $P < 0.001$ for both). The amount of all individual VFA was quadratically increased compared with NPN treatment for both CA ($P < 0.001$ to 0.006) and SP ($P < 0.001$ to 0.045), whereas acetate:propionate ratio was quadratically decreased ($P = 0.021$ and 0.047 for CA and SP, respectively). The amount of OMAF was increased (linear, $P = 0.027$ and 0.016 for CA and SP, respectively; quadratic, $P = 0.002$ and 0.014), whereas $\text{NH}_3\text{-N}$ concentrations were reduced by supplementing both protein-N sources (quadratic for CA, $P = 0.035$; linear, $P = 0.002$, and quadratic, $P < 0.001$ for SP).

Table 10. Effects of replacing non-protein nitrogen (NPN) by casein on in vitro fermentations in the absence of substrate in batch cultures of mixed ruminal micro-organisms incubated for 16.5 h*

Item	Treatment ¹			SEM	<i>P</i> -value [†]	
	BNPN	BCA50	BCA100		L	C
Gas (μmol)	342	387	546	33.5	0.377	0.005
CH ₄ (μmol)	90.3	116.6	140.2	4.76	0.008	0.001
Volatile fatty acid (μmol)						
Total	100	226	342	22.6	0.008	<0.001
Acetate	70.0	129	182	13.0	0.018	0.002
Propionate	8.5	30.0	54.8	3.61	0.006	<0.001
Butyrate	6.20	16.1	35.0	4.11	0.139	0.003
Isobutyrate	2.99	11.6	17.0	0.743	<0.001	<0.001
Isovalerate	10.6	26.4	36.6	1.13	<0.001	<0.001
Valerate	1.83	12.2	17.1	2.001	0.010	0.006
Acetate/Propionate (mol/mol)	9.39	4.29	3.30	0.930	0.008	0.021
CH ₄ /VFA (mol/mol)	0.930	0.516	0.410	0.1173	0.012	0.044
Organic matter apparently fermented (mg)	7.14	15.5	24.8	2.03	0.027	0.002
NH ₃ -N (mg/l)	331	332	268	19.1	0.972	0.035

* BNPN, BCA50 and BCA100: treatments whereby ammonia-N (BNPN; NH₄Cl) was replaced by casein-N at levels 0, 50 and 100% of total N, respectively.

[†] Linear (L) and quadratic (C) effects of casein.

Table 11. Effects of replacing non-protein nitrogen (NPN) by purified soybean protein on the in vitro fermentations in the absence of substrate in batch cultures of mixed ruminal micro-organisms incubated for 16.5 h*

Item	Treatment ¹			SEM ³	<i>P</i> -value [†]	
	BNPN	BSP50	BSP100		L	C
Gas (μmol)	342	333	340	16.6	0.714	0.906
CH ₄ (μmol)	90.3	101.8	116.6	2.53	0.019	<0.001
Volatile fatty acid (μmol)						
Total	100	146	198	5.0	<0.001	<0.001
Acetate	70.0	96.9	123	4.42	0.005	<0.001
Propionate	8.5	15.3	27.9	1.73	0.031	<0.001
Butyrate	6.20	8.95	11.17	0.534	0.008	<0.001
Isobutyrate	2.99	5.95	8.65	1.160	0.144	0.045
Isovalerate	10.6	15.8	20.0	0.737	0.003	<0.001
Valerate	1.83	3.43	7.52	0.606	0.057	<0.001
Acetate/Propionate (mol/mol)	9.39	6.25	4.61	1.050	0.079	0.047
CH ₄ /VFA (mol/mol)	0.930	0.697	0.589	0.0937	0.029	0.108
Organic matter apparently fermented (mg)	7.14	11.5	13.2	0.92	0.016	0.014
NH ₃ -N (mg/l)	331	283	182	6.6	0.002	<0.001

* BNPN, BSP50 and BSP100: treatments whereby ammonia-N (BNPN; NH₄Cl) was replaced by soybean-N at levels 0, 50 and 100% of total N, respectively.

[†] Linear effect (L) and quadratic (C) of soybean protein

3.4. DISCUSSION

Two synthetic substrates with different rate of degradation were used to analyze the possible interaction between the type of substrate and the N source on CH₄ production and substrate fermentation. Because all N treatments were applied at a rate considered adequate for optimal microbial growth (ARC, 1984) and minor VFA required for the growth of cellulolytic bacteria were added to the incubation medium, it was assumed that there were no limitations for the growth of ruminal bacteria. The NH₃-N in the incubation medium (mixture of buffer plus ruminal fluid) were similar each of the incubation days (39.3, 37.5, 34.3 and 36.7 mg NH₃-N/l for sheep 1, 2, 3 and 4, respectively). In addition, N:S ratio was lower than 10:1 in all N-treatments, thus preventing a limitation of S for the synthesis of S-containing amino acids by ruminal microbes (Ørskov, 1982).

The increases of CH₄ production observed by replacing the NPN by protein-N were higher for STAR (120 and 132 µmol/culture for CA and SP, respectively; values averaged across substitution levels) than for CEL substrate (63 and 37 µmol/culture, respectively). In contrast, the increases in VFA production were lower for STAR (125 and 78 µmol/culture for CA and SP, respectively; values averaged across substitution levels) compared with CEL (216 and 141 µmol per culture). The differences in the response to the supply of protein-N between STAR and CEL substrates resulted in significant N source x substrate interactions for CH₄/VFA ratio. The ratio CH₄/VFA can be used as an indicator of efficiency of ruminal fermentation, as CH₄ is an energy loss to the animal and VFA are used as an energy source and as substrates for the synthesis of other compounds. Whereas providing protein-N enhanced CH₄/total VFA ratio with STAR, the opposite was observed with CEL. These results might indicate that under conditions of limited energy availability, carbon skeletons resulting from amino acid deamination are mainly fermented to VFA, whereas amino acids can be used for microbial protein synthesis at a higher degree when energy is available.

Differences between substrates in the availability of methyl groups due to variable protein degradation and amino acid composition may have also contributed to the observed differences in CH₄ production, as methanogens of the order Thermoplasmatales that use methyl groups rather than H₂ have been identified in the rumen (Kim et al., 2011; Poulsen *et al.*, 2013). It should be noticed that the *in vitro* experiments conducted in this experiment lasted for 16.5 h, and some methanogens have greater half-lives (Hook et al., 2010); therefore, long-term *in vitro* studies are necessary to confirm these results.

Only few studies have compared the effects of the N source on CH₄ production and all have been conducted with fibrous substrates. Carro and Miller (1999) observed a 10.1% increase in CH₄ production by replacing half of the NPN by SP in RUSITEC fermenters receiving an all-fibre diet, which is quite well in agreement with the 7.7% of increase observed in the present study for the SP50 treatment and CEL substrate. Carro *et al.* (1999b) reported lower increases in CH₄ production (4.3 to 6.9%) in batch cultures with low-quality forages by replacing all NNP (urea) by casein than the 38.5% of increase observed in the present study for the CA100 treatment with CEL substrate. However, the substrates used in the study by Carro *et al.* (1999b) contained protein-N (67 to 78 g/kg of DM) and urea or casein were added up only to reach 30 mg of degradable N/g OM apparently fermented, whereas in the present study substrates and incubation medium were N-free and all the N was supplied by the experimental treatments.

The results from the batch cultures with the N sources as the only substrate also showed that the fermentation of CA and SP resulted in CH₄ production, with is in agreement with studies in cattle reporting that protein supplementation of forage-based diets increased CH₄ production (de Oliveria *et al.*, 2007; Sherk *et al.*, 2015). Blaxter and Martin (1962) measured CH₄ production in sheep fed dried grass and observed that infusing daily 15 to 31 g of Casein-N into the rumen increased CH₄ emissions by 1.82±0.275 mol per g of casein

infused. This value is higher than the 1.24 ± 0.172 mol per g of added CA observed in the present study, but also revealed a great variability between individual sheep. The two sheep used in the study of Blaxter and Martin (1962) gave values of 2.12 and 1.18 mol of CH₄ per g of added CA, whereas the values for the four inocula used in our study were 0.55, 1.18, 1.52 and 1.72 mol of CH₄ per g of CA. The great variability in CH₄ production per g of CA supports previous observations on the considerable variation in the amount of CH₄ produced by individual sheep (Pinares-Patiño *et al.*, 2003). The lower amounts of CH₄ produced from fermentation of SP (0.35, 0.58, 0.60 and 0.89 mol of CH₄ per g of SP for the four inocula used in the study) compared with those for CA might be due to an incomplete fermentation of SP or to a greater incorporation of SP-amino acids into microbial protein. The lower values of gas, CH₄ and VFA production, as well as the lower NH₃-N concentrations, observed for SP compared with CA support the hypothesis of an incomplete fermentation of SP after 16.5 h of incubation. Griswold and Mackie (1997) also reported lower ammonia-N concentrations at different incubation times (from 0 to 12 h) when *Prevotella ruminicola*, the most numerous *proteolytic bacterium* in the rumen, was grown in batch cultures with SP compared with CA as the only source of N. In our study ammonia-N concentrations were only measured at the end of the incubation, but were well above the concentration limiting *in vitro* microbial growth (Satter and Slyter, 1974) in all treatments.

The progressive replacement of NPN by protein-N also led to a progressive reduction of the acetate:propionate ratio from abnormally high values (9.39) in vials with only NPN to physiologically normal values in vials with only protein-N (3.30 and 4.61 for CA and SP, respectively). The shift of this ratio by increasing the supply of protein-N may indicate a predominance of acetate-producing microbes in conditions of limited availability of substrate (just endogenous substrate added with the inoculum in NPN-vials), as well as a proliferation of propionate-producing microbes when protein was available.

The higher increases in VFA production by supplying protein-N observed for CEL may indicate a more marked positive effect of protein-N on ruminal fermentation in situations of limited energy for microbial growth than in those with a rapidly available energy source (STAR substrate). Other *in vivo* (Wang *et al.*, 2013) and *in vitro* experiments with fermenters (Merry *et al.*, 1990; Molina-Alcaide *et al.*, 1996; Carro and Miller, 1999) or batch cultures (Carro *et al.*, 1999^{b, c}; Ranilla *et al.*, 2001) and fibrous diets have also showed that the replacement of NPN by amino acids, peptides, or protein-N resulted in increased in VFA production. All together these results indicate a stimulating effect produced by protein-N on the fermentation, presumably due to changes in bacterial populations affected by the form of N or to changes in its activity. Wang *et al.* (2013) observed that the ruminal infusion of soybean small peptides to cattle significantly increased the relative DNA abundance of *Butyrivibrio fibrisolvens* and decreased that of *Streptococcus bovis*, thus confirming changes in bacterial populations. In addition, the supply of fermentable OM in the form of protein-N could also have contributed to the observed increase in VFA production, as the amino acids resulting from proteolysis can be deaminated and the generated carbon skeletons be fermented to VFA (Ørskov, 1982). This fact is also shown by the increased VFA production with the progressive substitution of NPN by protein-N observed in the cultures with the N sources as the only substrate. The increased production of isobutyrate, isovalerate and valerate observed for both substrates by supplying CA and SP was expected, as these three VFA are major end products of amino acid fermentation (Wallace and Cotta, 1988). The decrease in NH₃-N concentrations by supplying protein-N is difficult to interpret, as concentrations of NH₃-N are the result of protein degradation and microbial protein synthesis processes. An increase in microbial protein synthesis has been observed *in vivo* and *in vitro* by replacing NPN by protein-N (Chikunya *et al.*, 1996; Carro and Miller, 1999; Ranilla *et al.*,

2001) and this may have contributed to the lower concentrations of $\text{NH}_3\text{-N}$ observed in the cultures receiving protein-N.

The amount of CH_4 produced is positively related to the amount of OM fermented (Johnson and Johnson, 1995), and the higher amount of OM fermented for STAR compared with CEL is in accordance with the higher amount of CH_4 generated in STAR fermentation. The lower values of total and individual VFA production observed for CEL compared with STAR agree well with the expected lower fermentability of CEL substrate. However, the lack of differences between substrates in the acetate/propionate ratio contrasts with the usual decrease in acetate/propionate ratio observed by increasing the amount of starch in the diet (Carro *et al.*, 2000). The high buffer capacity of the culture medium can partly explain this result, as final pH values were adequate in all cultures for an optimal activity of the cellulolytic flora (Stewart, 1977). Besides, the same inoculum was used for both substrates although it has been shown that the type of diet fed to donors is an important factor influencing the *in vitro* fermentation parameters (Martínez *et al.*, 2010a; Mateos *et al.*, 2013). The donor sheep were fed just one diet to avoid any additional factor of variability in the comparison of experimental treatments. Moreover, the *in vitro* system used in this study has a fixed substrate' retention time (16.5 h in our experiment), whereas retention time in the rumen is affected by feed characteristics.

3.5. CONCLUSIONS

The results indicate that the progressive replacement of NPN by protein-N increased both CH_4 and VFA production *in vitro*, but the magnitude of the changes was affected by the fermentability of the basal substrate. Whereas the increases in CH_4 emissions were higher for the rapidly fermented substrate than for the slowly-fermented one, the opposite was observed for the VFA production. These differences in the response to the supply of protein-N were attributed to differences in the use of the amino acids resulting from protein degradation and

possibly to changes in the microbial populations. If these results are confirmed *in vivo*, avoiding an excess of degradable protein in ruminant diets would not only to decrease the urinary excretion of N, but also the CH₄ emissions. In addition, the present study confirms the high individual variability in CH₄ production under *in vitro* conditions.

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CHAPTER 4.-

PROTECTION OF SUNFLOWER SEED AND SUNFLOWER MEAL PROTEIN WITH MALIC ACID AND HEAT: EFFECTS ON *IN VITRO* RUMINAL FERMENTATION AND METHANE PRODUCTION

**Jorge Leonardo Vanegas, María Dolores Carro, María Remedios Alvir and
Javier González**

*Departamento de Producción Agraria, Escuela Técnica Superior de Ingenieros
Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid,
Spain*

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4.1. INTRODUCTION

Reducing environmental impacts of animal production is currently an important goal of nutritionists. In this context, ruminants have a low efficiency of N use compared with non-ruminants and about 70-75% of the N ingested is excreted in manure (Huhtanen and Hristov, 2009), this inefficiency being attributable primarily to an inefficient use of N in the rumen, although post-absorptive N utilization is also inefficient. A reduction in the ruminal protein degradation of high-quality proteins can increase protein utilization and reduce N environmental emissions. The treatment of protein-rich feeds with heat and/or acid solutions has proven to be useful for protecting proteins from microbial degradation in the rumen, but the efficacy of these treatments varies with different factors, such as the type and concentration of acid and the heat intensity and duration (Broderick *et al.*, 1991). Previous *in vitro* and *in situ* studies have demonstrated the efficacy of a combined treatment with malic acid and heat to increase the protein value of sunflower meal (SM) and spring peas (Arroyo *et al.*, 2011; Díaz-Royón *et al.*, 2016).

Malic acid is converted to propionate in the rumen via fumarate and therefore can act as an electron sink for hydrogen and decrease the hydrogen availability for methanogenesis. In fact, malic acid and its sodium salts have been shown to stimulate propionate formation and inhibit methanogenesis in some *in vitro* studies (Martin and Streeter, 1995; Tejido *et al.*, 2005), although no clear reductions of methane emissions were found in others (Carro *et al.*, 1999a; Gómez *et al.*, 2005). In addition, the fermentation of the carbon chains resulting from amino acid deamination can contribute to the hydrogen supply to methanogenic archaea (Carro and Miller, 1999; Carro *et al.*, 1999b). Based on these results, our hypothesis was that the combined treatment with malic acid and heat for protein protection may not only reduce protein degradability, but may also improve the efficiency of ruminal fermentation by increasing propionate production and reducing methane emissions. The aim of this study was

therefore to investigate the effects of treating sunflower seeds (SS) and SM with malic acid at high-temperature on their *in vitro* fermentation and methane emissions. These two protein feeds were selected because they are extensively used in practical feeding of ruminants and differ in their protein degradability, which is usually higher in SS than in SM (Sauvant *et al.*, 2002).

4.2. MATERIALS AND METHODS

4.2.1. *Animals* and feeding

Four adult rumen-fistulated sheep (64.9 ± 2.04 kg body weight) were used as rumen fluid donors for the *in vitro* incubations. Animals were housed in individual pens with free access to water and a mineral-vitamin mixture Cl, Na, P, Ca, Mg, I, K, Zn, Mn, S, Fe, Co, Mo, Se and vitamins A, D3, E and niacin; Capriovi Produccion, Diaz del Prado Nutricion y Salud Animal, Talavera de la Reina, Spain). Sheep were fed a 2:1 mixed diet of lucerne hay and a commercial concentrate at energy maintenance level (ARC, 1984) distributed in two equal meals. The diet contained 913, 168, 426 and 269 g of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) per kg of dry matter (DM), respectively. Sheep management and rumen content withdrawal were carried out in accordance with the Spanish guidelines for experimental animal protection (BOE, 53/2013) in line with the European regulations. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Polytechnic University of Madrid.

4.2.2. *Feed samples and in vitro incubations*

One sample of SS (oil type) and one sample of semi-dehulled SM were used in the study. Five hundred grams of each of each substrate were weighed and pulverized with a solution of 1 M malic acid at a rate of 400 ml per kg of feed and left at room temperature for 1 h. Samples were then dried at 150°C for either 1 (MAL1) or 3 h (MAL3). The concentration

of malic acid and the duration of the heat treatment were selected from the results of previous studies (Arroyo *et al.*, 2011; Arroyo *et al.*, 2013). The chemical composition of the untreated (UNT) and treated samples is shown in Table 12.

Table 12. Chemical composition (g/kg dry matter unless otherwise stated) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 1 h (MAL1) or 3 h (MAL3)

Item	Sunflower seed			Sunflower meal		
	UNT	MAL1	MAL3	UNT	MAL1	MAL3
Dry matter (g/kg fresh matter)	977	980	971	919	969	998
Organic matter	969	970	969	927	932	930
Crude protein	179	171	172	359	337	343
Ether extract	467	459	318	12.9	20.2	15.9
Neutral detergent fibre	292	271	533	490	466	633
Acid detergent fibre	159	147	319	275	261	275
Acid detergent lignin	53.8	48.7	186	88.2	84.5	110
NDIN $\times 6.25^a$	54.3 (0.303)	42.6 (0.249)	144 (0.834)	89.8 (0.249)	98.3 (0.292)	246 (0.718)
ADIN $\times 6.25^a$	8.74 (0.049)	7.77 (0.046)	47.2 (0.274)	17.5 (0.049)	16.5 (0.049)	46.8 (0.137)

^a NDIN and ADIN: insoluble N in neutral and acid detergent solutions, respectively. Values in brackets are expressed as g/g of total crude protein.

Two different incubations were carried out and in each of them there were four replicates per feed and treatment. The first incubation was conducted to assess the gas production kinetics of the samples, whereas the main fermentation parameters were determined in the second incubation. Samples (500 and 300 mg of dry matter in the first and second incubation, respectively) of each substrate were carefully weighed into 120-ml serum bottles. The amount of sample in the second incubation was reduced to avoid high headspace

pressures that could have a detrimental effect on ruminal fermentation, as the gas produced was only released at the end of the incubation period. Ruminal contents from each sheep were obtained immediately before the morning feeding and strained through four layers of cheesecloth. The fluid of each sheep was then mixed independently with a buffer solution in a 1:4 ratio (vol/vol) at 39°C under continuous flushing with CO₂. The medium of Goering and Van Soest (1970) was modified by replacing the (NH₄)HCO₃ with NaHCO₃ and excluding the trypticase, and the resulting N-free solution was used as buffer. Bottles were prewarmed (39°C) prior to the addition of 50 ml of buffered rumen contents under CO₂ flushing. Then, bottles were sealed with rubber stoppers and aluminium caps and incubated at 39°C.

In the first incubation the gas production was measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a calibrated syringe at 3, 6, 12, 15, 20, 25, 30, 38, 48, 60, 72, 96, 120 and 144 h and the gas produced was released after each measurement. A total of 24 bottles (one per substrate and inoculum) were used. Additional bottles without substrate (blanks; two per inoculum) were included to correct the gas production values for gas release from endogenous substrates. After measuring the gas produced at 144 h of incubation, the fermentation was stopped by swirling the bottles in iced water, the bottles were opened and their content was transferred to previously weighed filter crucibles (pore size 100-160 µm) and filtered under vacuum. The residue of incubation was washed with 50 ml of hot distilled water, dried at 50°C for 48 h for estimating the apparent disappearance of dry matter after 144 h of incubation (DMD144).

The second incubation analyzed the effects of the treatments on the *in vitro* fermentation parameters. The incubation procedure was as before described with the exception that bottles contained 300 mg of substrate DM and were filled with 30 ml of buffered ruminal fluid. Two bottles per substrate and inoculum were incubated for 16.5 h,

value corresponding to a mean passage rate from the rumen of 0.06 per h. After 16.5 h, total gas production was measured as described before and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of methane. Bottles were then uncapped and the pH was measured immediately with a pH meter (Crison Basic 20, Crisson Instruments, Barcelona, Spain). Three millilitres of content was added to 3 ml of deproteinising solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per litre) for volatile fatty acid (VFA) determination and 2 ml were added to 2 ml 0.5 M HCl for $\text{NH}_3\text{-N}$ analysis. Finally, the content of all the bottles were filtered through crucibles, oven-dried at 50 °C and weighed to determine the disappearance of DM (DMD). Half of the crucibles (one per each feed, treatment and inoculum) were ashed (550°C for 8 h) to estimate OM disappearance (OMD). The residues of the other half of crucibles were analysed for NDF to estimate NDF disappearance (NDFD).

4.2.3. Chemical analyses

Dry matter (ID 934.01), ash (ID 942.05) and N (ID 984.13) contents were determined according to AOAC (1999). The analysis of NDF, ADF and acid detergent lignin (ADL) were carried out according to Van Soest *et al.* (1991) using an ANKOM220 Fibre Analyzer unit (ANKOMTechnology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF, ADF and ADL, and they were expressed exclusive of residual ash. Concentrations of $\text{NH}_3\text{-N}$ were determined using a spectrophotometer by the method of phenol-hypochlorite as previously described (Weatherburn, 1967) and those of VFA by gas chromatography as described by Carro *et al.* (1992). Analysis of methane was carried out following the procedure of Martínez *et al.* (2010) using a gas chromatograph (Shimadzu GC 14B; Shimadzu Europa GmbH, Duisburg, Germany) equipped with a flame ionization detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain).

4.2.4. Calculations and statistical analyses

In order to estimate the fermentation kinetic parameters in the first incubation, gas production data were fitted to the exponential model: $\text{gas} = A (1 - e^{-c(t-lag)})$, where A is the asymptotic gas production, c is the fractional rate of gas production, lag is the initial delay in the onset of gas production and t is the time of gas measurement. The parameters A , c and lag were estimated by an iterative least squares procedure using the NLIN procedures of SAS (2008 version 9.2; SAS Inst. Inc., Cary, NC, USA). The half time of gas production ($T_{1/2}$) was the time (h) when half of the asymptotic gas volume (A ; mL) was produced and was calculated as $T_{1/2} = [(\ln 2 / c) + lag]$. The average gas production rate (AGPR; mL gas/h) was defined as the average gas production rate between the start of the incubation and $T_{1/2}$, and was calculated as $AGPR = A c / [2 (\ln 2 + c lag)]$. Finally, the DM effective degradability (DMED) was estimated assuming a rumen particulate outflow (Kp) of 0.06 per h according to the equation: $DMED = [(DMD_{144} c) / (c + Kp)] e^{(-c lag)}$.

In the 16.5-h incubation, the amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation. The volume of gas produced was corrected for temperature (273°K; 0°C) and pressure (1 atm) and the amount of methane was calculated by multiplying gas produced by the methane concentration in the analysed sample. The amount of OM apparently fermented (OMAF) in each culture was estimated from acetate, propionate and butyrate production as described by Demeyer (1991). Values measured of the two bottles incubated for each inoculum, feed and experimental treatments were averaged before statistical analysis (4 values per feed and experimental treatment).

Accumulative gas production data were analysed independently for SS and SM as a mixed model with repeated measures using the PROC MIXED of SAS. The effects of the treatment, time and the interaction treatment x time were considered fixed and that of the

inoculum was random. The rest of the data were analysed independently for each feed as a mixed model using the PROC MIXED of SAS and considering the effect of the treatment fixed and that of the inoculum as random. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend. Comparison of means was performed by the Tukey test.

4.3. RESULTS AND DISCUSSION

4.3.1. Effects of treatments on chemical composition of samples

The chemical composition of raw SS and SM samples was in the range reported in the literature (NRC, 2001; Sauvant *et al.*, 2002), and the MAL1 treatment caused only minor changes in chemical composition of both protein feeds (Table 12). The slight decreases observed in CP, NDF, ADF and ADL content in the untreated samples compared with those MAL1-treated may be due to a dilution effect associated with adding malic acid in the protective treatment. The determination of ADIN has been proposed as a parameter to determine the extent of heat damage, as overheating may lead to the formation of indigestible compounds via the Maillard reactions between sugar aldehyde groups and free amino groups (Goering *et al.*, 1972; Broderick *et al.*, 1991). The fact that MAL1 treatment did not increase the concentration of ADIN in SS and SM, either expressed as g/kg DM or as percent of CP content, would indicate that this treatment was not severe enough to produce SS or SM protein damage. In contrast, the MAL3 treatment produced marked changes in the chemical composition of both feeds. The amount of NDF, ADF and ADL was 1.8, 2.0 and 3.5 times greater in MAL3 than in the raw samples for SS, respectively, and 1.3, 1.0 and 1.2 times for SM. In addition, both NDIN and ADIN content increased markedly as a consequence of MAL3 treatment, with values 2.7 and 5.4 times greater than those in the untreated SS sample, respectively, and 2.7 times greater in SM for both NDIN and ADIN. Despite these changes in NDIN and ADIN fractions, total CP content was not markedly affected by MAL3 treatment.

Similar results have been reported for other rich-protein feeds (Vanhatalo *et al.*, 1995; Pereira *et al.*, 1998), indicating that heat treatment did not produce total N loss. The actual effects of acid and heat treatment on protein are influenced mainly by acid concentration, pH, heating time, temperature and moisture (Broderick *et al.*, 1991; Pereira *et al.*, 1998; Arroyo *et al.*, 2013), although differences in susceptibility of different feeds have been reported to be large (Menke and Steingass, 1988; Mustafa *et al.*, 2003). Due to the impact of heat in feeds previously processed, such as the SM, the increases in NDF and ADF observed after heat treatment are usually greater in raw samples than in samples previously heated, which is in agreement with the higher increases observed in SS compared with SM in the present study. In addition, the large fat content of SS may have produced a “frying effect” that increased these reactions. The increases in fibre content observed in some studies have been greater for NDF than for ADF (Pereira *et al.*, 1998; Mustafa *et al.*, 2003), which is in agreement with our results. The MAL3 treatment produced a marked decrease in the ether extract content of SS, which was partly attributed to physical losses, as fat residues were observed in the drying trays when removing the sample after its treatment, although some fat combustion cannot be discarded.

4.3.2. *Effects of treatments on gas production kinetics*

The gas production was chosen to test the effects of the treatments because is a simple technique to evaluate substrate degradation, as the amount of gas produced is assumed to be directly proportional to substrate degradation (Menke and Steingass, 1988). The effects of the protective treatments on gas production values and the parameters of gas production kinetics are shown in Figure 7 and Table 13, respectively. For both feeds, gas production values for MAL3-treated samples were lower ($P < 0.05$) than those for untreated and MAL1 samples at all measured times, which indicates a reduced fermentation of MAL3 samples. These results are in accordance with the greater content of NDF and ADL observed in MAL3-treated SS

and SM samples compared with the untreated and MAL1 samples. In agreement with the results of gas production, the values of *A*, *c*, and AGPR were 0.58, 0.38 and 0.24 of those for untreated SS and 0.67, 0.53 and 0.36 of those for untreated SM, respectively ($P < 0.05$). Moreover, the DMED of MAL3 samples was decreased ($P < 0.05$) by 78 and 46% compared with the values for untreated SS and SM, respectively. All these results indicate that the MAL3 treatment reduced the amount of substrate available to the microbes for degradation, possibly by the formation of indigestible lignin-like polymers and phenolic compounds that are formed by heating and drying at high temperatures (Van Soest and Mason, 1991).

Figure 7. Gas production kinetics of sunflower seed (A) and sunflower meal (B) samples untreated (UNT; circles) and treated with malic acid at 150°C for 1 h (MAL1; squares) or 3 h (MAL3; triangles). Values of SEM and P of the ANOVA were 3.63 and $P < 0.001$ for SS and 3.16 and $P < 0.001$ for SM, respectively. For both samples MAL3 had lower ($P < 0.05$) gas production than UNT and MAL1 at all incubation times. Differences between UNT and MAL1 are indicated by † $P < 0.10$, * $P < 0.05$ and ** $P < 0.01$.

Table 13. Parameters of in vitro gas production kinetics and dry matter effective degradability (DMED) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 1 h (MAL1) or 3 h (MAL3)

Item	Sunflower seed					Sunflower meal				
	UNT	MAL1	MAL3	SEM	<i>P</i> =	UNT	MAL1	MAL3	SEM	<i>P</i> =
A (ml/g DM)	96.3 ^b	96.5 ^b	55.7 ^a	1.79	<0.001	168 ^b	181 ^b	113 ^a	3.83	<0.001
c (/h)	0.0896 ^b	0.0779 ^b	0.0341 ^a	0.00471	<0.001	0.0577 ^b	0.0535 ^b	0.0307 ^a	0.00180	<0.001
Lag (h)	1.17	1.20	0.25	0.311	0.113	0.11	0.00	0.00	0.053	0.298
AGPR (ml/h)	5.39 ^b	4.75 ^b	1.30 ^a	0.367	<0.001	6.85 ^b	6.93 ^b	2.48 ^a	0.223	<0.001
T _{1/2} (h)	9.08 ^a	10.4 ^a	26.5 ^b	4.14	0.044	12.3 ^b	13.3 ^b	23.0 ^a	0.65	<0.001
DMED (g/g)	0.325 ^c	0.259 ^b	0.073 ^a	0.0100	<0.001	0.334 ^b	0.322 ^b	0.179 ^a	0.0524	<0.001

^{a, b, c} Within each feed, means in the same row with different superscripts differ (*P*<0.05)

A: asymptotic gas production (ml/g DM); c: fractional rate of gas production (/h); Lag: initial time delay in the onset of gas production; AGPR: average gas production rate; T_{1/2}: time at which *half* of A is reached; DMED: Dry matter effective degradability for a rumen particulate outflow of 0.06

There were no differences ($P > 0.05$) in the amount of gas produced between untreated and MAL1 samples at any time for SS, but the fermentation of MAL1-treated SM produced greater amounts of gas than that of the untreated SM from the 38 h until the end of the incubation ($P < 0.10$ at 38 h; $P < 0.05$ at 48, 60 and 72 h; $P < 0.10$ at 96, 120 and 144 h). These results indicate a different response to MAL1 treatment for the two tested feeds, which is in agreement with the differences observed for other protein-rich feeds treated with heat (Plegge *et al.*, 1985; Vanhatalo *et al.*, 1995). The greater gas production of MAL1-treated SM might be due to the direct fermentation of the added malic acid, as malic acid is rapidly fermented by rumen microorganisms and amounts similar to that used in the present study were fermented *in vitro* within the first 12 h of incubation (Russell and Van Soest, 1984). However, the fact that the increase in gas production for MAL1 treatment was first noticed at 38 h of incubation would indicate that other mechanisms are involved in the observed response. In addition, a hypothetical rapid fermentation of malic acid would have decreased the lag time for MAL1 treatment, but no differences ($P > 0.05$) between treatments were observed in this parameter for any feed. For both SS and SM, there were no differences ($P > 0.05$) between untreated and MAL1-treated samples in any gas production kinetics parameter (A , c , AGPR and $T_{1/2}$), indicating that this treatment did not cause negative effects on substrate degradation. The MAL1 treatment of SS decreased ($P < 0.05$) DMED of from 0.325 to 0.259, due to numerical reductions in the rate of gas production (0.0896 and 0.0779 h^{-1} for untreated and MAL1 samples, respectively), but no effects ($P > 0.05$) were observed for SM (0.334 and 0.322 for untreated and MAL1 samples, respectively).

4.3.3. Effects of treatments on *in vitro* fermentation

As shown in Tables 14 and 15, the results of the 16.5 h incubation confirmed the negative effects of MAL3 treatment on substrate degradability, as it reduced ($P < 0.05$) the production of gas and VFA by 28 and 42% for SS and by 27 and 38% for SM, respectively.

The MAL3 treatment also reduced ($P < 0.05$) the methane emission by 45 and 44% for SS and SM, respectively. The amount of malic acid incubated with the feed per bottle (120 μmol) had a potential decrease in methane production of 30 μmol according to the stoichiometric equation $\text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$, which is much lower than the reduction of 81 and 142 μmol of methane per bottle observed for MAL3 treatment for SS and SM, respectively. Therefore, this reduction was attributed to decreased fermentable substrate rather than to a direct effect on methanogenesis, as indicated by the lower ($P < 0.05$) amount of OMAF and the lower values of DMD and OMD observed for MAL3-treated samples. The MAL3-treated SM had lower NDFD values than the untreated samples (Table 15), which is consistent with the reduced methane production due to the close relationship between the amount of fibre fermented and that of methane generated (Johnson and Johnson, 1995). The extremely low values (close to 0; values not shown) of NDFD of SS for all treatments was attributed to the high ether extract content of SS, as the free fatty acids resulting from the triglyceride hydrolysis are toxic for the fibrolytic bacteria (Maia *et al.*, 2007).

Compared with the untreated samples, the MAL3 treatment increased ($P < 0.05$) molar proportion of acetate for both feeds, but effects on other VFA were variable. Treating the SS with MAL3 resulted in lower ($P < 0.05$) proportions of propionate, butyrate, valerate and higher ($P < 0.05$) caproate proportions, whereas the treatment of SM decreased the proportion of isovalerate and valerate without affecting the propionate, butyrate, isobutyrate and caproate proportions. These results indicate again a great variability between feeds in the response to acid-heat treatments.

Table 14. Effects of the treatment of sunflower seed with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 1 h (MAL1) or 3 h (MAL3) on *in vitro* fermentation and dry matter (DMD) and organic matter disappearance (OMD) in batch cultures of sheep rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n=4)

Item	Treatment			SEM	P =
	UNT	MAL1	MAL3		
Gas (µmol)	1541 ^b	1564 ^b	1113 ^a	54.1	0.002
CH ₄ (µmol)	233 ^c	197 ^b	152 ^a	10.4	0.005
Proportion of CH ₄ in gas	0.155	0.127	0.140	0.0074	0.099
NH ₃ -N (mg/l)	302 ^c	222 ^b	122 ^a	4.64	<0.001
Total volatile fatty acids	784 ^b	731 ^b	456 ^a	31.6	<0.001
Molar proportion (mol/100 mol)					
Acetate (Ac)	59.2 ^a	58.9 ^a	64.7 ^b	0.37	<0.001
Propionate (Pr)	19.5 ^b	23.7 ^c	16.2 ^a	0.55	<0.001
Butyrate	9.93 ^b	8.16 ^a	8.65 ^{ab}	0.377	0.039
Isobutyrate	4.03	3.70	3.92	0.139	0.295
Isovalerate	4.33	3.35	3.81	0.243	0.084
Valerate	2.53 ^c	2.05 ^b	1.83 ^a	0.062	<0.001
Caproate	0.50 ^a	0.15 ^a	0.89 ^b	0.134	0.023
Ac/Pr (mol/mol)	3.05 ^b	2.53 ^a	4.00 ^c	0.067	0.001
CH ₄ /VFA (mol/mol)	0.300	0.269	0.333	0.0310	0.049
Organic matter fermented (mg)	62.8 ^b	58.8 ^b	34.2 ^a	2.58	<0.001
DMD (g/g)	0.346 ^c	0.269 ^b	0.123 ^a	0.0130	<0.001
OMD (g/g)	0.355 ^c	0.294 ^b	0.137 ^a	0.0148	<0.001

^{a, b, c} Means in the same row with different superscripts differ (P < 0.05)

UNT: untreated sample. Values of organic matter fermented were estimated from volatile fatty acids production according to Demeyer (1991). Values for neutral detergent fibre degradability were close to 0 for all treatments and are not reported.

Table 15. Effects of the treatment of sunflower meal with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 1 h (MAL1) or 3 h (MAL3) on *in vitro* fermentation and disappearance of dry matter (DMD), organic matter (OMD) and neutral detergent fibre (NDFD) in batch cultures of sheep rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n=4)

Item	Treatment			SEM	P =
	UNT	MAL1	MAL3		
Gas (µmol)	2173 ^b	2240 ^b	1578 ^a	15.2	<0.001
CH ₄ (µmol)	320 ^c	284 ^b	178 ^a	6.81	<0.001
Proportion of CH ₄ in gas (mol/mol)	0.148 ^c	0.127 ^b	0.112 ^a	0.00353	0.001
NH ₃ -N (mg/l)	303 ^c	259 ^b	147 ^a	5.07	<0.001
Total volatile fatty acids	1220 ^b	1276 ^b	756 ^a	26.0	<0.001
Molar proportion					
Acetate (Ac)	62.8 ^a	64.3 ^a	67.8 ^b	0.45	0.004
Propionate (Pr)	18.4 ^a	21.8 ^b	18.4 ^a	0.26	<0.001
Butyrate	8.22	7.48	7.68	0.27	0.199
Isobutyrate	2.83 ^b	2.58 ^a	2.83 ^b	0.059	0.039
Isovalerate	2.73 ^b	2.16 ^a	1.93 ^a	0.074	<0.001
Valerate	1.75 ^c	1.53 ^b	1.18 ^a	0.066	0.003
Caproate	0.32	0.23	0.16	0.070	0.354
Ac/Pr (mol/mol)	3.61 ^b	2.96 ^a	3.69 ^b	0.069	<0.001
CH ₄ /VFA (mol/mol)	0.262 ^b	0.224 ^a	0.235 ^a	0.0078	0.029
Organic matter fermented	99.6 ^b	104.7 ^b	62.4 ^a	2.19	<0.001
DMD (g/g)	0.344 ^b	0.333 ^b	0.171 ^a	0.0071	<0.001
OMD (g/g)	0.373 ^b	0.357 ^b	0.188 ^a	0.0188	<0.001
NDFD (g/g)	0.259 ^c	0.189 ^b	0.043 ^a	0.0194	<0.001

^{a, b, c} Means in the same row with different superscripts differ (P < 0.05)

UNT: untreated sample. Values of organic matter fermented were estimated from volatile fatty acids production according to Demeyer (1991).

In agreement with the results of the gas production trial, the MAL1 treatment of SS and SM did not negatively affect the production of gas and total VFA and the amount of OMAF indicating that this treatment did not reduce the substrate fermentability. The average reduction of 36 μmol of methane per bottle observed for MAL1 treatment for both feeds is slightly greater than the potential decrease in methane production of 30 μmol calculated for the amount of malic acid added per bottle. However, in a meta-analysis of malate effects on methanogenesis in ruminal batch cultures, Ungerfeld and Forster (2011) observed that methane decrease with malic acid has generally been quite lower than expected according to stoichiometry calculations. In fact, the MAL1 treatment decreased methane production by 15.5 and 11.3% for SS and SM, respectively, values greater than the 7–8% decrease in methane formation found by others (Carro and Ranilla, 2003a; Tejido *et al.*, 2005; Ungerfeld and Forster, 2011) in ruminal batch cultures for similar initial concentrations of malic acid or malate salts and suggest that methane reductions in the present study cannot be exclusively attributed to the fermentation of malate itself. The observed reduction in NDFD of MAL1-treated SM compared with raw SS would support this hypothesis, as the degradation of NDF usually results in high proportions of acetate and butyrate and the formation of both VFA requires the production of CO_2 and hydrogen which are used by the methanogenic archaea to form CH_4 (Van Soest, 1994). The lower OMD observed for MAL1-treated SS compared with untreated SS is also in agreement with this hypothesis. In addition, the fermentation of protein has been shown to contribute to methane generation (Carro and Miller, 1999; Carro *et al.*, 1999b) and a decrease in protein fermentation in MAL1-treated samples is supported by the lower ($P < 0.05$) ammonia-N concentrations observed for both feeds and by the reduced molar proportions of isobutyrate, isovalerate and valerate, which are generated in the degradation of branched-chain amino acids. The MAL1 treatment also increased ($P < 0.05$) the molar proportions of propionate for both feeds and decreased that of butyrate ($P < 0.05$)

for SS. The 0.45 mol/mol recovery of malic acid as propionate observed for the SM agrees well with the average value of 0.48 mol/mol reported by Ungerfeld and Forster (1995) but the recovery for SS was much lower (0.17 mol/mol), indicating differences between feeds. The effects of malic acid and malate salts on VFA profile have been reported to vary with the incubated substrate (Carro and Ranilla, 2003a; Tejido *et al.*, 2005), but an increase in the propionate production has been observed in most *in vitro* studies because malate is an intermediate of the succinate pathway of propionate production in the rumen (Gómez *et al.*, 2005). The observed increase in propionate production is in agreement with the reduction in the production of methane, as the generation of propionate incorporates reducing equivalents (Gómez *et al.*, 2005). Due to these changes, the MAL1 treatment reduced ($P < 0.05$) the acetate/propionate ratio for both feeds and decreased the ratio methane/VFA from 0.300 to 0.269 for SS and from 0.262 to 0.224 for SM, differences being only significant ($P < 0.05$) for SM. These results indicate an improvement in the efficiency of ruminal fermentation and could imply in greater energy supply for the host animal.

The lower ($P < 0.05$) DMD and OMD values observed in MAL1-treated SS compared with untreated SS are in accordance with the lower DMED of SS estimated in the gas production trial (22.2, 17.2 and 20.3% of reduction, respectively) and may be due to a reduction in the substrate degradation rate, as previously discussed. Thus, values of *c* were numerically lower for MAL-1 than for the untreated SS samples, although the differences did not reach the significance level ($P = 0.129$). This slow degradation rate may also affect the ruminal fermentation of the unsaturated fat of this SS. Therefore, studies investigating the effects of this treatment on the biohydrogenation of SS fatty acids and its subsequent intestinal digestibility would be of interest to evaluate the actual increase of its nutritive value. Finally, it should be noticed that the amount of DM and OM digested and incorporated into microbial biomass has not been taken into account in any of the fermentation variables

measured, and studies determining the microbial protein synthesis with protein protected feeds are needed.

4.4. CONCLUSIONS

The results indicate that the MAL1 treatment is effective at protecting a highly degradable protein rich in sulphur-containing amino acids and tryptophan such as sunflower protein from the ruminal degradation. In addition, this treatment improved the efficiency of sunflower seed and sunflower meal fermentation by decreasing the emission of methane and increasing the proportion of propionate without negatively affecting total VFA production. However, overprotection by excessive heat duration (150 °C for 3 h) should be avoided because it reduced the fermentability of both feeds and led to protein damage as indicated by the increased NDIN and ADIN fractions.

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CHAPTER 5.-

INFLUENCE OF MALIC ACID ON IN VITRO RUMEN FERMENTATION OF SUNFLOWER PRODUCTS: A COMPARISON OF ITS USE AS A PROTECTIVE AGENT AGAINST PROTEIN DEGRADATION AND AS A FEED ADDITIVE

**Jorge Leonardo Vanegas, Javier González, María Remedios Alvir and María
Dolores Carro**

*Departamento de Producción Agraria, Escuela Técnica Superior de Ingeniería Agraria,
Agroalimentaria y de Biosistemas, Universidad Politécnica de Madrid, Ciudad Universitaria,
28040 Madrid, Spain.*

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5.1. INTRODUCTION

Reducing protein degradation in the rumen can both increase digestive utilization of protein and help to reduce N emissions. A recent study demonstrated the efficacy of a combined treatment with malic acid and heat to reduce the protein degradation of sunflower seed (SS) and sunflower meal (SM) and to improve the in vitro fermentation by lowering CH₄ emissions and increasing propionate production without affecting total volatile fatty acids (VFA) production (Vanegas *et al.*, 2016). However, it is unknown whether the effects were due to the protective effect of the combined malic acid-heat treatment, to malic acid fermentation itself, or even to a combination of both factors.

Some studies have evidenced the influence of the diet of donor animals on CH₄ production (Martínez *et al.*, 2010a) and on the effectiveness of additives to modify ruminal fermentation (Kamel *et al.*, 2008; Mateos *et al.*, 2013). As highlighted by Mould *et al.* (2005), diet characteristics and nutrients intake are major factors affecting both the microbial populations in the rumen and the activity of the ruminal fluid used as inoculum for in vitro incubations. The effectiveness of malic acid as feed additive to modify the in vitro rumen fermentation has been shown to vary with the incubated substrate (Carro and Ranilla, 2003; Gómez *et al.*, 2005), but to our knowledge the possible influence of the donors' diet has not yet been assessed. Therefore, the aim of this study was to compare the in vitro fermentation of malic acid-heat treated SS and SM with that of the untreated feeds, either supplemented with malic acid or unsupplemented, when rumen fluid from sheep fed two contrasting diets was used as inoculum. Our hypothesis was that the effects of the acid malic-heat treatment of both protein feeds on their in vitro rumen fermentation would depend on microbial populations in the inoculum, and therefore on the type of diet fed to the host animal. In addition, the effects of supplementing SS and SM with either malic acid or disodium malate were compared, as the chemical form of malate supply (free acid vs. salts) has been proposed

as a factor involved in the variability of the response to malate supplementation observed in different studies (Carro and Ungerfeld, 2015). To our knowledge the in vitro fermentation of the two forms of malate has not yet being compared.

5.2. MATERIALS AND METHODS

5.2.1. Feeds, animals and feeding

One sample of SS (oil type) and one sample of semi-dehulled SM were used in the study. Samples each substrate (500 g) were weighed and pulverized with a solution of 1 M malic acid at a rate of 400 ml per kg of feed, left at room temperature for 1 h and subsequently dried at 150°C for 1, as described by Vanegas *et al.* (2016). The chemical composition of the untreated (UNT) and malic acid-heat treated (MAH) samples is shown in Table 16.

Table 16. Chemical composition (g/kg dry matter unless otherwise stated) of sunflower seed and sunflower meal either untreated (UNT) or treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 1 h (MAH) used as substrates for in vitro incubations and of diets fed to rumen fluid donor sheep¹.

Item	Sunflower seed		Sunflower meal		Donor diets	
	UNT	MAH	UNT	MAH	MC	HC
Dry matter (g/kg fresh matter)	977	980	919	969	901	900
Organic matter	969	970	927	932	909	900
Crude protein	179	171	359	337	152	149
Ether extract	467	459	12.9	20.2	31.5	33.7
Neutral detergent fibre	292	271	490	466	408	364
Acid detergent fibre	159	147	275	261	222	191
Acid detergent lignin	53.8	48.7	88.2	84.5	34.4	31.3
NDIN × 6.25	54.3 (0.303)	42.6 (0.249)	89.8 (0.249)	98.3 (0.292)	ND	ND
ADIN × 6.25	8.74 (0.049)	7.77 (0.046)	17.5 (0.049)	16.5 (0.049)	ND	ND

¹ MC and HC: medium and high concentrate diets, respectively. NDIN and ADIN: N insoluble in neutral and acid detergent solutions, respectively. Values in brackets are expressed as g/g of total crude protein. ND: not determined

Four adult rumen-fistulated sheep (64.7 ± 2.10 kg body weight) were used as rumen fluid donors for the *in vitro* incubations. Animals were housed in individual pens with free access to water and a mineral-vitamin mixture. Sheep were fed two different diets in a cross-over experimental design with two 15-d periods. The medium-concentrate diet (MC) was composed of 30:20:50 triticale hay:alfalfa hay: concentrate, and the high-concentrate diet (HC) was composed of 15:85 barley straw:concentrate (Table 16). The concentrate was composed by wheat middlings, sunflower meal, barley grain, rye grain, palm kernel cake, calcium carbonate, rapeseed meal, tallow, sugarbeet molasses, sodium chloride and mineral/vitamin premix in proportions of 249, 180, 169, 150, 82.1, 23.6, 15.1, 10.0, 10.0, 7.8 and 4.0 g/kg fresh matter, respectively. Forage:concentrate ratios in the diets were representative of those for medium-lactation animals (MC) and fattening ruminants under intensive systems of production (HC). The diets were fed at a fixed rate of 42 g of dry matter (DM) per kg of body weight^{0.75} distributed in two equal meals. Sheep management and rumen contents sampling were carried out in accordance with the Spanish guidelines for experimental animal protection (BOE, 53/2013) in line with the European regulations. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the Polytechnic University of Madrid.

5.2.2. *In vitro* incubations

Samples (300 mg of DM) of each UNT and MAH samples were carefully weighed into 120-ml serum bottles. Additionally, UNT samples (283 mg of DM) were weighed in bottles which received 1ml of a solution containing either 17 mg of malic acid (**MA** treatment) or 22.6 mg of disodium malate (**DSM** treatment) immediately before incubation. This was done to supply the same amount of malate in the MAH samples, either in the form of free acid or disodium salt. Bottles from UNT and MAH treatments received 1ml of distilled water to equal the liquid volume. In addition, bottles without substrate and receiving 1 ml of distilled

water, 1 ml of the MA solution, or 1 ml of the DSM solution were incubated for analysing the fermentation of both additives as the only substrate.

The last day of each experimental period, rumen content from each donor sheep was obtained immediately before the morning feeding and strained through 4 layers of cheesecloth. Three ml of fluid was added to 3 ml of deproteinising solution (20 g of metaphosphoric acid and 0.6 g of crotonic acid per litre) for volatile fatty acids (VFA) determination, and 2 ml were mixed with 2 ml 0.5 M HCl for $\text{NH}_3\text{-N}$ analysis. The fluid from each sheep was individually mixed with a buffer solution in a 1:4 ratio (vol/vol) at 39°C under continuous flushing with CO_2 . The medium of Goering and Van Soest (1970) was modified by replacing the $(\text{NH}_4)\text{HCO}_3$ with NaNCO_3 and excluding the trypticase to obtain a N-free buffer. Bottles were prewarmed (39°C) prior to the addition of 30 ml of the mixture under CO_2 flushing. Finally, bottles were sealed with rubber stoppers and incubated at 39°C for 16.5 h. Four bottles were incubated for each inoculum, feed, and experimental treatment.

After 6 h of incubation, the gas production was measured in half of the bottles using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe, and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH_4 . Bottles were uncapped and the pH was measured immediately (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain). Bottles were placed in iced water to slow down fermentation and samples of the content were taken for VFA and $\text{NH}_3\text{-N}$ analyses as described before. After 16.5 h of incubation (corresponding to a mean passage rate from the rumen of 0.06 per h), the rest of the bottles were processed and sampled as described previously. Two bottles per inoculum (MC and HC), feed (SS and SM), incubation time (6 and 16.5 h), and experimental treatment (UNT, TR, MA and DSM) were incubated in each of the experimental periods.

5.2.3. Chemical analyses

Dry matter (ID 934.01), ash (ID 942.05) and N (ID 984.13) contents were determined according to the Association of Official Analytical Chemists (1999). The analysis of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were carried out according to Van Soest *et al.* (1991) using an ANKOM220 Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in the sequential analysis of NDF, ADF and ADL, and they were expressed exclusive of residual ash. Concentrations of $\text{NH}_3\text{-N}$ were determined by the method of phenol-hypochlorite as previously detailed (Weatherburn, 1967). Analyses of VFA and CH_4 were conducted by gas chromatography as described by Carro *et al.* (1992) and Martínez *et al.* (2010a), respectively.

5.2.4. Calculations and statistical analyses

The amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation. The amount of OM apparently fermented (OMAF) in each culture was estimated from VFA production as described by Demeyer (1991). Values measured of the two bottles incubated for each inoculum, feed, sampling time and experimental treatment were averaged before statistical analysis to get four replicates per treatment.

Data from each incubation time were analysed independently for SS and SM as a mixed model using the PROC MIXED of SAS. The effects of the experimental treatment, donors' diet and the interaction treatment x diet were considered fixed, and that of the inoculum was random. The sums of squares were further partitioned by orthogonal contrasts to analyze differences among treatments. The contrasts were distributed as follows, C1: UNT versus MAH; C2: MAH versus MA; C3: UNT versus MA and DSM; C4: MA versus DSM. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend.

5.3. RESULTS AND DISCUSSION

5.3.1. Influence of malic acid-heat treatment

As shown in Table 16, the MAH treatment caused only minor changes in chemical composition of SS and SM. The slight decreases observed in crude protein, NDF, ADF and ADL content in the MAH samples compared with those in the UNT samples were attributed to a dilution effect by the addition of malic acid in the protective treatment (Vanegas *et al.*, 2016). The MAH treatment did not increase the concentration of acid detergent insoluble N in any feed, which would indicate that no protein damage was produced (Goering *et al.*, 1972; Pereira *et al.*, 1998).

The effects of experimental treatments on in vitro fermentation at 6 and 16.5 h of incubation are shown in Tables 17 and 18 for SS and in Tables 19 and 20 for SM, respectively. Compared with UNT, the MAH treatment of SS decreased ($P < 0.001$) the production of gas, CH₄, total and individual VFA production, as well as the amount of OMAF, at both incubation times, excepting the propionate production at 16.5 h ($P = 0.923$). These results indicate a reduction of SS fermentation caused by MAH treatment. The lack of differences on propionate production was attributed to malic acid fermentation itself, as malic acid is mainly metabolized to propionate in the rumen (Carro and Ungerfeld, 2015).

Table 17. Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower seed in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 6 h (n=4)¹

Item	Treatment				Donor diet		P =						Donor Diet	Treatment x Donor diet
							Treatment ²							
	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4			
Gas (μmol)	1217	1005	1389	1162	1256	1131	38.5	<0.001	<0.001	0.090	<0.00	<0.001	0.851	
CH ₄ (μmol)	196	93.0	192	187	160	174	11.4	<0.001	<0.001	0.554	0.653	0.115	0.818	
NH ₃ -N (mg/l)	192	96.4	191	180	170	160	7.6	<0.001	<0.001	0.348	0.186	0.082	0.923	
Volatile fatty acids (VFA; μmol)														
Total VFA	623	385	715	670	613	544	25.4	<0.001	<0.001	0.005	0.088	<0.001	0.382	
Acetate (Ac)	367	213	384	372	373	294	15.2	<0.001	<0.001	0.436	0.447	<0.001	0.822	
Propionate (Pr) ³	132	115	205	195	172	151	5.3	<0.001	<0.001	<0.00	0.066	<0.001	0.006	
Butyrate	81.9	38.7	83.9	67.3	73.3	62.6	7.32	<0.001	<0.001	0.328	0.034	0.054	0.621	
Isobutyrate	13.5	6.26	13.8	12.1	11.7	11.2	1.09	<0.001	<0.001	0.547	0.128	0.557	0.828	
Isovalerate	18.5	8.20	18.6	15.8	14.4	16.1	1.22	<0.001	<0.001	0.235	0.031	0.065	0.677	
Valerate	9.53	4.11	10.3	8.10	7.91	8.13	0.693	<0.001	<0.001	0.616	0.004	0.651	0.142	
Ac/Pr (mol/mol)	2.76	1.84	1.86	1.89	2.18	1.99	0.113	<0.001	0.781	<0.00	0.738	0.007	0.714	
CH ₄ /VFA (mol/mol) ³	0.325	0.259	0.278	0.291	0.246	0.331	0.019	0.002	0.330	0.021	0.472	<0.001	0.035	
OMAF (mg) ⁴	53.7	32.8	61.3	56.8	56.1	46.2	2.47	<0.001	<0.001	0.022	0.086	<0.001	0.432	

¹ UNT: untreated; TR: treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet

² Orthogonal contrasts: C1: UNT vs MAH; C2: MAH vs MA; C3: UNT vs MA and DSM; C4: MA vs DSM

³ Propionate production values for UNT, MAH, MA and DSM were 140, 138, 206 and 206 µmol for MC-diet and 124, 92.7, 204 and 184 µmol for HC-diet, respectively. CH₄/VFA values for UNT, MAH, MA and DSM were 0.275, 0.183, 0.261 and 0.263 mol/mol for MC-diet and 0.376, 0.332, 0.294 and 0.319 mol/mol for HC-diet, respectively.

⁴ OMF: organic matter fermented estimated from volatile fatty acids production according to Demeyer (1991)

Table 18. Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower seed in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n=4)¹

Item							P =					Donor Diet	Treatment x Donor diet
	Treatment				Donor diet		Treatment ²						
	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4		
Gas (μmol)	2126	1611	2371	2072	1960	2130	114.9	<0.001	<0.001	0.349	0.017	0.049	0.928
CH ₄ (μmol)	400	159	389	388	336	332	24.8	<0.001	<0.001	0.577	0.966	0.835	0.968
NH ₃ -N (mg/l)	364	199	373	351	295	348	16.9	<0.001	<0.001	0.874	0.196	<0.001	0.651
Volatile fatty acids (VFA; μmol)													
Total VFA	1246	1048	1323	1280	1325	1119	43.5	<0.001	<0.001	0.200	0.236	<0.001	0.633
Acetate (Ac)	734	596	744	708	753	639	30.4	<0.001	<0.001	0.760	0.242	<0.001	0.941
Propionate (Pr)	258	259	325	321	299	282	11.3	0.923	<0.001	<0.001	0.736	0.051	0.131
Butyrate	157	129	161	159	182	116	5.2	<0.001	<0.001	0.594	0.629	<0.001	0.523
Isobutyrate	25.8	18.5	26.8	25.2	25.5	22.7	1.32	<0.001	<0.001	0.902	0.276	0.009	0.775
Isovalerate	27.0	17.3	25.3	26.1	25.2	22.4	2.53	<0.001	<0.001	0.176	0.808	0.006	0.628
Valerate	44.3	26.0	40.6	41.3	39.6	36.5	1.38	<0.001	<0.001	0.143	0.778	0.101	0.422
Ac/Pr (mol/mol)	2.94	2.30	2.33	2.22	2.53	2.36	0.114	<0.001	0.803	<0.001	0.341	0.044	0.737
CH ₄ /VFA (mol/mol)	0.330	0.161	0.302	0.310	0.255	0.297	0.0227	<0.001	<0.001	0.253	0.704	0.015	0.857
OMAF (mg) ³	106	90.3	113	107	115	93.4	3.51	<0.001	<0.001	0.178	0.151	<0.001	0.630

¹ UNT: untreated; TR: treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet

² Orthogonal contrasts: C1: UNT vs MAH; C2: MAH vs MA; C3: UNT vs MA and DSM; C4: MA vs DSM

³ OMF: organic matter fermented estimated from volatile fatty acids production according to Demeyer (1991)¹⁶

Table 19. Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower meal in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 6 h (n=4) ¹

Item	Treatment				Donor diet		P =					Donor Diet	Treatment x Donor diet
							Treatment ²						
	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4		
Gas (μmol)	1400	1501	1473	1338	1533	1323	61.0	0.117	0.660	0.926	0.038	<0.001	0.953
CH ₄ (μmol)	250	204	239	230	256	211	15.7	0.020	0.220	0.349	0.641	0.004	0.316
NH ₃ -N (mg/l)	158	133	136	150	157	132	3.7	<0.001	0.398	<0.001	0.002	<0.001	0.780
Volatile fatty acids (VFA;													
Total VFA	724	758	781	789	847	679	29.9	0.216	0.408	0.016	0.756	<0.001	0.935
Acetate (Ac)	479	473	474	480	541	412	15.4	0.666	0.941	0.851	0.755	<0.001	0.602
Propionate (Pr)	142	198	214	211	202	181	6.2	<0.001	0.019	<0.001	0.608	<0.001	0.988
Butyrate	68.3	61.9	65.1	66.0	72.8	57.9	7.57	0.407	0.682	0.675	0.902	0.012	0.862
Isobutyrate	6.85	5.56	6.09	6.74	6.78	5.84	0.839	0.004	0.199	0.215	0.115	0.003	0.810
Isovalerate	12.2	9.74	10.5	12.1	11.7	10.5	1.23	0.008	0.360	0.224	0.085	0.049	0.763
Valerate	14.5	9.96	11.3	13.8	13.0	11.8	0.395	0.002	0.291	0.089	0.056	0.184	0.244
Ac/Pr (mol/mol)	3.36	2.37	2.20	2.27	2.75	2.34	0.059	<0.001	0.009	<0.001	0.256	<0.001	0.391
CH ₄ /VFA (mol/mol)	0.338	0.269	0.310	0.253	0.301	0.282	0.0314	0.035	0.179	0.052	0.082	0.394	0.209
OMAF (mg) ³	61.4	64.4	66.2	66.6	72.0	57.4	2.53	0.259	0.470	0.034	0.880	<0.001	0.977

¹ UNT: untreated; TR: treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet

² Orthogonal contrasts: C1: UNT vs MAH; C2: MAH vs MA; C3: UNT vs MA and DSM; C4: MA vs DSM

³ O MF: organic matter fermented estimated from volatile fatty acids production according to Demeyer (1991)

Table 20. Effects of the experimental treatments and diet of donor sheep on *in vitro* fermentation of sunflower meal in batch cultures of rumen microorganisms containing 300 mg of substrate dry matter and incubated for 16.5 h (n=4) ¹

Item	Treatment				Donor diet		P =						
							Treatment ²					Donor Diet	Treatment x Donor diet
	UNT	MAH	MA	DSM	MC	HC	SEM	C1	C2	C3	C4		
Gas (μmol)	2455	2559	2507	2389	2401	2554	115.6	0.383	0.661	0.943	0.321	0.076	0.996
CH ₄ (μmol)	501	367	524	470	469	463	23.9	<0.001	<0.001	0.850	0.035	0.732	0.117
NH ₃ -N (mg/l)	325	269	280	308	278	314	10.9	<0.001	0.337	0.004	0.018	<0.001	0.803
Volatile fatty acids (VFA; μmol)													
Total VFA	1470	1456	1550	1557	1408	1605	25.3	0.441	<0.001	0.001	0.762	<0.001	0.899
Acetate (Ac)	903	882	913	927	862	951	22.2	0.358	0.181	0.398	0.550	<0.001	0.902
Propionate (Pr)	278	315	361	350	303	349	11.0	0.003	<0.001	<0.001	0.323	<0.001	0.920
Butyrate	188	186	186	185	163	206	3.0	0.215	0.501	0.351	0.780	<0.001	0.939
Isobutyrate	25.8	19.9	24.9	25.1	21.9	26.0	0.99	0.003	0.011	0.600	0.890	0.004	0.460
Isovalerate	28.5	20.4	26.6	27.1	23.4	26.9	2.09	<0.001	<0.001	0.061	0.223	<0.001	0.990
Valerate	45.8	31.8	42.0	43.3	35.3	44.9	1.79	<0.001	0.001	0.027	0.199	<0.001	0.915
Ac/Pr (mol/mol)	3.25	2.80	2.55	2.65	2.87	2.76	0.112	<0.001	0.043	<0.001	0.391	0.183	0.880
CH ₄ /VFA (mol/mol) ³	0.343	0.255	0.340	0.275	0.304	0.287	0.0190	<0.001	<0.001	0.046	0.003	0.241	0.006
OMAF (mg) ⁴	126	126	133	133	121	139	1.93	0.995	0.001	<0.001	0.990	<0.001	0.897

¹ UNT: untreated; TR: treated; MA: malic acid added; DSM: disodium malate added; MC: medium-concentrate diet; HC: high-concentrate diet

² Orthogonal contrasts: C1: UNT vs MAH; C2: MAH vs MA; C3: UNT vs MA and DSM; C4: MA vs DSM

³ CH₄/VFA values for UNT, MAH, MA and DSM were 0.328, 0.277, 0.325 and 0.286 mol/mol for MC-diet, and 0.358, 0.172, 0.356 and 0.263 mol/mol for HC-diet, respectively.

⁴ OMF: organic matter fermented estimated from volatile fatty acids production according to Demeyer (1991).

In contrast, no effects of MAH treatment either in the production of gas and total VFA or in the amount of OMAF were detected for SM at any incubation time, indicating that MAH had no negative effects on its dry matter degradation. In contrast, a decrease in protein degradation in the cultures with MAH-treated SM is supported by the lower ($P < 0.01$) $\text{NH}_3\text{-N}$ concentrations and molar proportions of isobutyrate, isovalerate and valerate, as these minor VFA are generated in the degradation of branched-chain amino acids. These results indicate a different response of SS and SM to MAH treatment, which is in agreement with the differences observed for other high-protein feeds treated with heat (Vanhatalo *et al.*, 1995; Mustafa *et al.*, 2003). Differences between feeds in the response to MAH treatment in the production of individual VFA were also observed. Whereas for SS the MAH treatment reduced ($P < 0.001$) the production of all individual VFA excepting that of propionate at 16.5 h incubation ($P = 0.923$), the MAH treatment of SM increased ($P < 0.01$) the production of propionate, reduced ($P < 0.01$) that of the minor VFA and did not change acetate and butyrate production.

The MAH treatment reduced CH_4 production ($P < 0.05$) and $\text{NH}_3\text{-N}$ concentrations ($P < 0.001$) for the two tested feeds at both incubation times, but the decreases were more pronounced for SS than for SM. Compared to UNT, $\text{NH}_3\text{-N}$ concentrations in MAH-cultures were decreased by 47.6 and 16.5% for SS and SM, respectively (values averaged across incubation times), and CH_4 production was reduced by 56.4 and 22.6%, respectively. As shown in Figure 8, positive correlations ($P < 0.001$) between $\text{NH}_3\text{-N}$ concentrations and CH_4 production in the fermentation of UNT and MAH samples were detected for both SS and SM. Positive correlations between $\text{NH}_3\text{-N}$ concentrations and CH_4 production have also been reported in other in vitro studies in which fermentations were conducted at different pH (Lana *et al.*, 1998) or using rumen fluid with different protozoa species as inoculum (Ranilla *et al.*, 2007). The relationships between $\text{NH}_3\text{-N}$ and CH_4 may just reflect the different amounts of

organic matter fermented in the cultures, but in the current study there were no differences in the OMAF between UNT and MAH-treated SM samples and still a highly significant correlation was observed. Therefore, our hypothesis was that MAH treatment affected mainly the protein fraction of SM, and the lower CH₄ production was at least partly due to reduced protein degradation. In agreement with this hypothesis, previous studies (Arroyo *et al.*, 2013; Díaz-Royón *et al.*, 2016) reported that only 10-15% of the by-pass fraction of SM treated with malic acid-heat was due to non-protein compounds. The lack of significant differences between UNT and MAH-treated SM in the OMAF in the present study is in agreement with these results. Protein degradation usually results in an increase in NH₃-N concentrations and the fermentation of the carbon chains resulting from amino acid deamination can contribute to the hydrogen supply to methanogenic archaea, thereby the decrease in protein degradation can result in lower NH₃-N concentrations and less CH₄ production as previously reported (Carro and Miller, 1999; Carro *et al.*, 1999b).

Figure 8. Relationship between ammonia-N concentrations and methane production in batch cultures containing sunflower seed (A) or sunflower meal (B), either untreated or treated with malic acid and heat, as substrates and incubated for 6 (empty symbols) and 16.5 (full symbols) hours. Batch cultures were inoculated with ruminal fluid from sheep fed medium-concentrate (triangles) or high-concentrate (circles) diets. Incubations were conducted in quadruplicate.

5.3.2. *Malic acid and disodium malate as feed additives*

For both feeds, MA and DSM treatments resulted in increased ($P < 0.02$) production of total VFA and propionate and reduced acetate/propionate ratios compared with UNT at both incubation times, with the exception of total VFA production at 16.5 h incubation for SS ($P = 0.200$). The values of added malate (120 μmol) recovered as propionate (0.61 and 0.56 mol/mol for MA and DSM, respectively; values averaged across feeds and incubation times) are slightly higher than the average value of 0.48 mol/mol reported by Ungerfeld and Forster (2011) in a review of in vitro studies. These results support studies reporting a rapid fermentation of both MA and DSM by rumen microorganisms, as propionate increases at 6 and 16.5 h were similar (73 vs. 75 μmol per culture for MA, respectively, and 66 vs. 68 μmol for DSM; values averaged across feeds) (Russell and Van Soest, 1984; Callaway and Martin, 1997). Compared with UNT, neither MA nor DSM treatments reduced CH_4 production ($P > 0.05$), which is consistent with the variable effects of malate supplementation on CH_4 production reported in other studies (Carro and Ranilla, 2003a; Carro and Ungerfeld, 2015). The lower ($P < 0.01$) $\text{NH}_3\text{-N}$ concentrations observed for MA and DSM treatments of SM compared with UNT were attributed to a greater $\text{NH}_3\text{-N}$ capture by ruminal microorganisms as a consequence of the greater fermentable organic matter supply as malate. In contrast, no differences ($P > 0.05$) in $\text{NH}_3\text{-N}$ concentrations among UNT, MA and DSM treatments were observed for SS, possibly due to the higher protein degradability in SS compared to that in SM (Sauvant *et al.*, 2002). The observed differences between SS and SM in the response to MA and DSM supplementation are in agreement the inconsistency reported in previous studies (Carro and Ranilla, 2003a; Carro and Ungerfeld, 2015).

Compared with the supply of DSM, adding MA to SS resulted in greater ($P < 0.05$) production of gas and tended ($P < 0.10$) to increase total VFA production and the amount of OMAF, but after 16.5 h of incubation, only differences in gas production were detected. A

different response was observed for SM, as MA treatment resulted in lower $\text{NH}_3\text{-N}$ concentrations ($P < 0.05$) at both incubation times, greater gas production ($P < 0.05$) at 6 h, and greater CH_4 production ($P < 0.05$) at 16.5 h compared with the supply of DSM. These results indicate a different response due to the form of malate supply (MA or DSM), which helps to explain the controversial results observed in the literature, mainly regarding the potential effects of malate on rumen methanogenesis (Carro and Ungerfeld, 2015; Ungerfeld and Forster, 2011). However, there were no differences ($P > 0.05$) in the VFA and CH_4 production in the batch cultures with MA and DSM as the only substrate either at 6 or 16.5 h of incubation (averaged values, 478 and 475 μmol VFA, and 151 and 151 μmol CH_4 for MA and DSM, respectively; results not shown). This indicates that MA and DSM were fermented similarly when they were the only substrate, but differently when they were added to a fermentable substrate (SS or SM).

In agreement with the reduced degradation observed for MAH-treated SS, the direct supply of MA to cultures containing SS resulted in greater ($P < 0.001$) production of gas, CH_4 and total and individual VFA compared with the MAH treatment at both incubation times. In contrast, there were no differences ($P > 0.05$) between MA and MAH treatments in the production of gas, CH_4 and total and individual VFA (excepting that of propionate which was higher for MA) for SM at 6 h incubation, showing that malic acid was fermented at a similar rate in both treatments, and that the MAH treatment did not depress the fermentation of SM. Nevertheless, after 16.5 h the cultures with MAH-treated SM had lower productions of both CH_4 and minor VFA than those with MA-treated SM, showing the greater efficacy of MAH treatment to reduce both protein degradation and CH_4 emissions compared with the use of MA as a feed additive.

5.3.3. Influence of donors' diet

The two types of inoculum used in this experiment differed in fermentation characteristics (results not shown). In accordance with previous studies in sheep (Carro *et al.*, 2000; Ramos *et al.*, 2009), the fluid from MC-fed sheep had greater ($P < 0.05$) pH (6.68 vs. 6.03), $\text{NH}_3\text{-N}$ concentrations (162 vs. 129 mg/l) and acetate:propionate ratio (4.72 vs. 4.39), but lower propionate proportion ($P = 0.042$; 14.0 vs. 15.4 mol/100 mol), compared with the HC-inoculum. These differences would indicate that feeding the two diets promoted different microbial communities in the rumen fluids.

The donors' diet influenced ($P < 0.05$) most of the measured parameters in the batch cultures. For both feeds, at 6 h of incubation the MC-inoculated cultures had greater ($P < 0.05$) gas and VFA production, acetate/propionate ratio and OMAF than those inoculated with HC-fluid. After 16.5 h of incubation, the MC-inoculated cultures had lower total VFA production than those HC-inoculated with SS as substrate, but the opposite was observed for cultures with SM. These results indicate that at short incubation times (6 h) the MC-inoculum had a higher degradative activity than the HC-inoculum, but at longer incubation times (16.5 h) the degradative activity differed with the fermented substrate. This was probably due to the adaptation of the microbial populations in the inoculum to the incubation conditions in the batch cultures (substrate, temperature, pH, etc.), as recently observed by Mateos *et al.* (2015).

Despite of the differences in the fermentation parameters due to the donors' diet, treatment x donors' diet interactions were only observed for propionate production ($P = 0.006$) and CH_4/VFA ratio ($P = 0.035$) at 6 h of fermentation for SS, and for CH_4/VFA ratio ($P = 0.006$) at 16.5 h of SM fermentation. Whereas the MAH treatment of SS decreased the propionate production at 6 h of incubation with the HC-inoculum (124 and 92.7 μmol for UNT and MAH, respectively), no changes were observed with the MC-inoculum (140 and 138 μmol for UNT and MAH). Similarly, the decrease in the CH_4/VFA ratio produced by the

MAH-treatment of SM at 16.5 h incubation was more pronounced for the HC-inoculum (0.358 and 0.172 mol/mol for UNT and MAH, respectively) than for the MC-inoculum (0.328 and 0.277 mol/mol). These results illustrate that the effectiveness of treatments to modify rumen fermentation may depend on the type of diet fed to donor animals, as previously observed for some feed additives (Kamel *et al.*, 2008; Mateos *et al.*, 2013).

5.4. CONCLUSIONS

The results indicate that the combined malic acid-heat treatment was effective at improving rumen fermentation, by protecting the sunflower protein from degradation and reducing CH₄ emissions and NH₃-N concentrations. This treatment was more effective than the use of the same amount of malic acid as feed additive. The positive relationship observed between CH₄ emissions and NH₃-N concentrations suggests that a decrease in protein degradation would result in less CH₄ production, highlighting the importance of avoiding an excess of degradable protein in the diet to reduce both CH₄ and N emissions. The differences in the response observed to either malic acid or disodium malate supplementation help to explain the controversial results observed in the literature regarding malate effects on in vitro rumen fermentation.

CHAPTER 6.-

GENERAL DISCUSSION

6.1. GENERAL DISCUSSION

The results of each experiment have been previously discussed in detail and therefore the objective of this chapter is to analyze all the results in a joint discussion.

6.1.1. *Ruminal degradation of cell wall-bounded nitrogenous compounds*

The protein evaluation models for ruminants have several limitations, but one of the most important is the lack of a precise system to determine the protein degradability in the rumen due to the microbial contamination of the undegraded feed particles in the *in situ* technique. The activity of the methanogenic archaea is closely related to the fibre degradation and therefore the degradation of the nitrogenous compounds associated to the cell wall might have a more important role in the CH₄ production compared with that of other proteins.

The microbial contamination of the fibrous fractions is high (Rodríguez and González, 2006) and a correction for the contribution of microbial N is necessary for an accurate protein evaluation. The use of the ¹⁵N dilution technique using ¹⁵N-enriched feeds has an important limitation that is the incorporation of ¹⁵N from the colonised ¹⁵N-enriched feed particles by the solid-associated bacteria (SAB). However, the results of previous studies (González *et al.*, 2012; Díaz-Royón, 2016) indicate that 20% of the total N in SAB comes from the colonized feed particles. The use of this average value allowed correcting the values of the degradation kinetics of several ¹⁵N-enriched feeds for the first time in the Experiment 1 of this Doctoral Thesis. The results of this study show that microbial N contamination of the nitrogenous fractions of the cell wall (NDIN and ADIN) is very high, not only for fibrous feeds but also for concentrates (wheat grain), making necessary a correction to obtain accurate values. The results agree with those of Arroyo and González (2013) and Guevara-González *et al.* (2015), confirming that the treatment of the residues with detergent solutions (neutral or acid) does not remove the microbial contamination. The results of this study clearly show that the sequential treatment with neutral and acid detergent removed only

partially the attached microbes. This makes more appropriate the use of the ^{15}N -enrichment values for NDIN and ADIN for the correction than using the ^{15}N -enrichment of the feed.

The ^{15}N -enrichment values observed for NDIN and ADIN fractions indicate that the ADIN content of feeds is usually overestimated due to absorption of N by the fibre particles during the boiling in the solution of cetyl-trimethylammonium bromide during the acid detergent treatment. Thus, the ^{15}N -enrichment values in the total-N and in the NDIN fraction were similar, but they were lower for the ADIN fraction for both the whole feed sample and its insoluble fraction. The reduction was low for the sunflower seed (SS; 1.28%), but important for wheat grain (WG) and wheat straw (WS), with values of 9.95 and 9.24%, respectively, which is in agreement with the differences in the fibre polarity of the tested feeds. Thus, the waxy cuticle and the high content in lignin and lipids in the SS hulls indicate a higher hydrophobicity of the ADF fraction of SS compared with that of WG and WS. This hypothesis is worth to be tested, as the ADIN fraction is closely related to the protein value of feeds. It has to be noticed that the sequential treatment with neutral and acid detergents was developed to isolate the lignocellulose residue of feeds and not the N associated to the cell wall. The possible N-absorption in the ADF residues might be the cause of the more rapid degradation of ADIN than that of NDIN observed for Arroyo and González (2013) for an Italian ryegrass hay and for WS in this study (k_d : 6.3 vs 41.7 %/h for NDIN and ADIN, respectively).

The ruminal degradability values of NDIN and ADIN corrected for the microbial contamination were high for the three tested feeds, but their values were lower than those for the total N. Similar results of those found for NDIN were reported for Arroyo and González (2013) and for most of the feeds tested by Guevara-González *et al.* (2015), although these last authors observed degradability values higher than those for total N for feeds with low-degradability proteins, such as dried distilled wheat grains or palm meal. No differences were

detected between the degradability values of NDIN and ADIN in this study, with the exception of the WG. Similar results were reported by Arroyo and González (2013) for a low-quality Italian ryegrass hay and by Guevara-González *et al.* (2015) for a medium-quality English ryegrass hay. In contrast, Guevara-González *et al.* (2015) observed differences in the NDIN and ADIN degradability for an oat hay of high degradability (71.8 vs 50.4%, respectively). The similar degradability values of NDIN and ADIN observed for SS and WS in our study and for ryegrass hay in the above cited studies indicate a similar rate of degradation of the tissues including both fractions, which are highly lignified. However, it should be considered that the possible N-absorption from the cetyl-trimethylammonium bromide into the ADF fraction produces an overestimation of the ADIN, producing an overvaluation of its degradability, and therefore reducing the difference between the NDIN and ADIN degradabilities.

The contribution of NDIN degradation to total degraded N observed in this study (7.25, 13.7 and 20.3% for SS, WG and WS, respectively) shows the importance of this N fraction in the polluting emissions generated in the fermentation process. The results of Guevara-González *et al.* (2015) for seven feeds showed also an important contribution, ranging from 9.65 to 81.9%. The contribution of ADIN degradation to total degraded N was low (2.69, 0.27 and 7.25% for SS, WG and WS, respectively), and was lower as the feed quality increased. Guevara-González *et al.* (2015) reported contributions of 3.46 and 2.71% for an English ryegrass hay and oat hay, respectively, and Arroyo and González (2013) observed a value of 11.5% for a low-quality Italian ryegrass hay. In the present study the calculation of degradability values was made taken into account only the rumen evacuation rate (5%/h) but not the comminution rate whereas in those of Arroyo and González (2013) and Guevara-González *et al.* (2015) these both rates were considered. The use of both rates for the calculation of degradability would result in higher values, especially for fibrous

highly-lignified feeds such as wheat straw or sunflower seed hulls, which also have low comminution rates.

6.1.2. Protein fermentation and *in vitro* methane production

The Experiments 2, 3 and 4 of this Doctoral Thesis were designed to investigate the contribution of protein fermentation to *in vitro* CH₄ production. As our hypothesis was that protein contribution might vary with the type of carbohydrate available to ruminal microbes, two pure substrates composed by carbohydrates of variable fermentation rate were used as basal substrates in the Experiment 2. In addition, two protein sources of different degradation rate (casein (CA) and soybean protein (SP)) were selected, and the incubation conditions (N supply, N:S ratio, supply of minor VFA) were carefully designed to avoid a limitation of microbial growth in experimental any treatment.

In agreement with the few studies investigating this topic (Carro and Miller, 1999; Carro *et al.*, 1999a), the replacing of NPN by protein-N resulted in increased CH₄ production, but the increases were higher for STAR than for CEL substrate (76 and 50 µmol/culture, respectively; values averaged across substitution levels). In contrast, the increases in VFA production observed by replacing NPN by protein-N were lower for STAR compared with CEL substrate (102 and 179 µmol/culture, respectively). These results would indicate that when the microorganisms have a supply of energy rapidly available (STAR substrate) the AA can be used for microbial protein synthesis, but conditions of limited energy availability (CEL substrate) results in higher AA deamination and the generated carbon skeletons are fermented to VFA. The results showed an intensification of fermentation with the progressive replacement of NPN by protein-N for both substrates, which is consistent with the incorporation of increasing amounts of fermentable organic substrates, presumably due to changes in density and composition of bacterial populations.

The results of the batch cultures with the N sources as single substrates also showed that the fermentation of CA and SP results in CH₄ production, and the obtained values (0.55, 1.18, 1.52 and 1.72 mol of CH₄ per g of CA, values for each individual inoculum) were in the range of those reported by Blaxter and Martin (1962) in sheep fed dried grass (2.12 and 1.18 mol of CH₄ per g of added CA, values for two individual sheep). The great variability in CH₄ production per g of CA observed in our study also supports previous observations on the considerable variation in the amount of CH₄ produced by individual sheep (Pinares-Patiño *et al.*, 2003; Martínez *et al.*, 2010b). The lower amounts of CH₄ produced from fermentation of SP (0.35, 0.58, 0.60 and 0.89 mol of CH₄ per g of SP values for each individual inoculum) compared with those for CA might be due to an incomplete fermentation of SP or to a greater incorporation of AA from SP into microbial protein; however, the lower values of gas, CH₄ and VFA production and NH₃-N concentrations observed for SP compared with CA support the hypothesis of an incomplete fermentation of SP after 16.5 h of incubation.

The decrease in NH₃-N concentrations observed for both substrates by replacing NPN by protein-N is difficult to interpret, as concentrations of NH₃-N in batch cultures are the result of protein degradation and microbial protein synthesis processes. The reduced NH₃-N concentrations in our study were probably due to an increased uptake of NH₃-N or amino acids by rumen microorganisms for microbial protein synthesis, as previous studies have shown an increase in microbial synthesis by replacing NPN by protein-N (Chikunya *et al.*, 1996; Carro and Miller, 1999; Ranilla *et al.*, 2001). However, an incomplete protein degradation cannot be discarded, especially for SP. In fact, the lower concentrations of NH₃-N observed for SP compared with CA supports the hypothesis of a rapid and complete degradation of CA by ruminal microorganisms and of an incomplete fermentation of SP after 16.5 h of incubation.

The Experiments 3 and 4 were also designed to investigate the contribution of protein fermentation to *in vitro* CH₄ production, but two protein concentrates were used as basal substrates. Sunflower seed (SS) and meal (SM) were chosen because they are widely used in ruminant feeding and differ in their fat content and protein degradability, which is higher for SS than for SM (Sauvant *et al.*, 2002). The treatment of protein-rich feeds with heat and/or acid solutions has proven to be useful for protecting proteins from microbial degradation in the rumen, and previous studies by our group (Arroyo *et al.*, 2011, 2013; Díaz-Royón *et al.*, 2016) showed the efficacy of a combined malic acid-heat treatment for protecting the protein of several concentrate feeds against ruminal degradation. As malic acid is converted to propionate in the rumen via fumarate and therefore can act as an electron sink for hydrogen and decrease the CH₄ formation, our hypothesis was that the combined malic acid-heat treatment could not only reduce the protein degradability, but also improve the efficiency of ruminal fermentation by increasing propionate production and reducing CH₄ emissions. However, the efficacy of the acid-heat treatments varies with different factors, such as the type and concentration of acid and the heat intensity and duration (Broderick *et al.*, 1991; Pereira *et al.*, 1998), and therefore two different heating periods were tested in Experiment 3: 150°C for 1 (MAL1) or 3 h (MAL3).

The MAL3 treatment produced marked changes in the chemical composition of both tested feeds, resulting in sharp increases in NDF, ADF, ADL, NDIN and ADIN fractions, but the total crude protein content remained unchanged, indicating that heat treatment not produced total N loss. Overheating can lead to the formation of indigestible compounds via the Maillard reactions between sugar aldehyde groups and free amino groups (Goering *et al.*, 1972; Broderick *et al.*, 1991). Therefore, the increased ADIN concentrations in the MAL3-treated samples (5.4 and 2.7 times greater than those in the untreated SS and SM samples, respectively) are indicative of protein damage. In agreement with this hypothesis, the MAL3-

treated samples had lower values of potential gas production, gas production rates and DM effective degradability (determined in 144-h *in vitro* incubations) compared with the untreated samples, indicating that this treatment reduced the amount of substrate available for microbial degradation. The negative effects of MAL3 treatment on substrate degradability were confirmed in 16.5-h incubations, as the production of VFA and CH₄ were markedly reduced (by 42 and 38% for SS and for SM, respectively for VFA, and by 45 and 44% for CH₄). All together these results show that the MAL3 treatment resulted in overheating and caused an excessive reduction of ruminal degradability.

In contrast, the MAL1 treatment caused only minor changes in chemical composition of both protein feeds, without increasing the ADIN concentration, which indicates that this treatment did not produce protein damage. The lack of differences between the untreated SS and SM and the MAL1-treated samples in any gas production kinetics parameter (*A*, *c*, AGPR and *T*_{1/2}) show that MAL1 treatment did not cause negative effects on substrate degradation. In accordance with this hypothesis, the MAL1 treatment of SS and SM did not affect either the production of total VFA or the amount of organic matter apparently fermented. However, this treatment reduced significantly the NH₃-N concentrations (by 26.5 and 11.3% for SS and SM, respectively), which is indicative of reduced protein degradation. The reduced protein degradation is also supported by the lower proportions of isobutyrate and isovalerate generated in the fermentation of MAL1-treated samples, as these minor VFA are formed in the degradation of branched-chain AA.

The amount of malic acid incubated with the feed per culture (120 μmol) had a potential decrease in methane production of 30 μmol according to the stoichiometric equation $\text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$, which is in well agreement with the reduction of 36 μmol of CH₄ per culture observed for both SS and SM. The slightly higher reduction observed compared with that predicted from the stoichiometry expected (36 vs. 30 μmol per culture)

might indicate that the CH₄ reduction was not exclusively due to the fermentation of malate itself. The observed reduction in NDF degradability of MAL1-treated SM compared with the untreated SM would support this hypothesis, as the degradation of NDF usually results in high proportions of acetate and butyrate and carries out an associated production of CO₂ and hydrogen, which are used by the methanogenic archaea to form CH₄ (Van Soest, 1994). The lower organic matter degradability observed for MAL1-treated SS compared with untreated SS is also in agreement with this hypothesis.

The evidences of the contribution of protein fermentation to CH₄ generation observed in Experiment 2 were also found in Experiment 3, as indicated by the positive correlation found between the NH₃-H concentrations and the CH₄ production for both SS ($r = 0.659$; $P = 0.020$; $n = 12$) and SM ($r = 0.864$; $P < 0.001$; $n = 12$). The MAL1 treatment also increased the molar proportions of propionate for both feeds. The 0.45 mol/mol recovery of malic acid as propionate observed for the SM agrees well with the average value of 0.48 mol/mol reported by Ungerfeld and Forster (1995), but the lower recovery for SS (0.17 mol/mol) indicates differences between feeds in utilization of the malic acid. These results agree with the variable effects of malic acid and malate salts on VFA profile reported in the literature (Carro and Ranilla, 2003a; Tejido *et al.*, 2005; Gómez *et al.*, 2005). The observed increase in propionate production is in agreement with the reduction in the production of CH₄, as the generation of propionate incorporates reducing equivalents (Gómez *et al.*, 2005).

The results of Experiment 3 indicate that the MAL1 treatment of SS and SM resulted in an improvement in the efficiency of ruminal fermentation and could imply in greater energy supply for the host animal. However, it was unknown whether the observed effects were due exclusively to the protective effect of the combined malic acid-heat treatment, to malic acid fermentation itself, or even to a combination of both factors. Therefore, the Experiment 4 was designed to compare the effects of the combined acid malic-heat treatment

with those of supplementing untreated SS and SM samples with the same dose of malate (either malic acid or disodium malate). Both malic acid and disodium malate were used because the chemical form of malate supply (free acid vs. salts) has been proposed as a factor involved in the variability of the response to malate supplementation observed in different studies (Carro and Ungerfeld, 2015). Moreover, based on previous studies (Mateos *et al.*, 2013) we hypothesized that the effects of the acid malic-heat treatment of SS and SM on their *in vitro* rumen fermentation would depend on the microbial populations in the inoculum, and therefore on the type of diet fed to the host animal. To test this hypothesis the donor sheep were fed two contrasting diets (medium-concentrate (MC) and high-concentrate (HC) diets), and their rumen fluids were used as inoculum for the *in vitro* incubations.

Similarly to that observed in Experiment 3, the MAL1 treatment reduced the $\text{NH}_3\text{-N}$ concentrations and CH_4 production for both feeds, but the reduction in CH_4 (by 52.6 and 60.3% for SS and SM, respectively) was higher than that detected in Experiment 3 (15.5 and 11.3% for SS and SM, respectively). The differences between experiments might be due to a higher degradative activity in the inoculum used in the Experiment 3, as the diet fed to donor sheep contained a high proportion of a good quality forage (66.6% of alfalfa hay) compared with the diets used in Experiment 4, which had both smaller proportions of forage and lower quality forage. The higher supply of nutrients and the higher rates of comminution and transit of the alfalfa hay compared with the forage mixture used in Experiment 4 should be reflected in a higher microbial density and activity (González *et al.*, 1987; Rodríguez *et al.*, 2008). In agreement with this hypothesis, previous studies have reported greater microbial concentrations and bacterial diversity in high-forage diets than in high-concentrate diets (Martínez *et al.*, 2010b), although these results can be affected by the quality of forages and concentrates. Moreover, the MAL1 treatment of SS caused a reduction of total VFA production that was not detected in Experiment 3, despite of using the same MAL1-treated

SS sample in both experiments. No effects of MAL1 treatment on total VFA production were observed for SM in any experiment. These results indicate that the *in vitro* fermentation of different substrates can be influenced by the type of inoculum in a different way.

Significant treatment $\times$ donors' diet interactions were only observed for propionate production and the CH₄/VFA ratio at 6 h of fermentation for SS, and for CH₄/VFA ratio at 16.5 h of SM fermentation. Whereas the MAH treatment of SS decreased the propionate production at 6 h of incubation with the HC-inoculum, no changes were observed with the MC-inoculum. Similarly, the decrease in the CH₄/VFA ratio produced by the MAH-treatment of SM at 16.5 h incubation was more pronounced for the HC-inoculum than for the MC-inoculum, indicating that the effectiveness of treatments to modify rumen fermentation may depend on the type of diet fed to donor animals, as previously observed for feed additives (Kamel *et al.*, 2008; Mateos *et al.*, 2013).

The supplementation of SS and SM with malic acid or disodium malate resulted in increased total VFA production, but was ineffective to reduce the CH₄ production, demonstrating the advantage of using malic acid in the protective treatment (malic acid-heat treatment). Supplementing the SS with malic acid or disodium malate had no effect on NH₃-N concentrations, but caused a reduction of NH₃-N concentrations for SM, which could have been due to a greater NH₃-N or AA capture by ruminal microorganisms for microbial protein synthesis as a consequence of the greater fermentable organic matter supply as malate.

The positive highly significant correlations observed between the NH₃-N concentrations and the CH₄ production in the fermentation of untreated and MAL1-treated samples for both SS and SM agree with the same observations in Experiment 3 and indicate that the reduction in CH₄ generation could be partly due to the reduced protein fermentation. The relationships between NH₃-N and CH₄ may just reflect different amounts of organic matter fermented, but in this study there were no differences in the organic matter fermented

between the cultures with untreated SM samples and those with MAL1-treated SM samples, and still a highly significant correlation was observed.

All together the results of this Doctoral Thesis indicate that a decrease in protein degradation in the rumen would result in less CH₄ production, highlighting the importance of avoiding an excess of degradable protein in the diet of ruminants to reduce both CH₄ and N emissions. However, care should be taken in ensuring the necessary supply of degradable protein in the diet to meet the N requirements of rumen microorganisms for an optimal growth and activity.

CHAPTER 7.-

CONCLUSIONS

7. CONCLUSIONS

Ruminal degradation of cell wall-bounded nitrogenous compounds

1. The decrease in ^{15}N abundance of the acid detergent insoluble nitrogen (ADIN) fraction of all tested samples indicates that ADIN content is over-evaluated due to N contamination with cetyl-trimethylammonium bromide in the procedure for ADF analysis. As ADIN fraction is frequently used in feed protein evaluation, further studies to clarify this point are needed.
2. The procedures for isolating neutral detergent fibre (NDF) and ADF fractions failed to remove large fractions of particle-associated microorganisms, and this produced an under-evaluation of the effective degradability of neutral detergent insoluble nitrogen (NDIN) and ADIN. Assuming that 20-21% of adherent microorganisms-N comes from the colonized feed, as proposed by González *et al.* (2012), allows to correct for microbial contamination using the ^{15}N dilution technique and to obtain more precise estimations of the effective degradability of NDIN and ADIN.
3. Ruminal degraded NDIN represented a significant part of the degraded N in all tested feeds. Contributions of ADIN degradation to total degraded N had some importance in the tested feeds, except for wheat grain in which was negligible

Protein fermentation and in vitro methane production

1. The progressive replacement of non-protein N (NPN) by protein-N increased both CH_4 and volatile fatty acid (VFA) production *in vitro*, but the final products varied with the fermentability of the basal substrate. With a rapidly-fermented substrate (starch-rich) the fermentation of the amino acids supplied by proteins was mainly oriented to CH_4 production, whereas with a slowly-fermented substrate (cellulose-rich) the increase in VFA production moderated the increase in the CH_4 production.

2. The effects of the replacement of NPN by protein-N on the *in vitro* fermentation efficiency measured by the CH₄/VFA ratio depended also on the fermentation rate of the basal substrate, as this ratio was increased for the starch-rich substrate and decreased for the cellulose-rich substrate. The results show that the fermentation of the amino acid-carbon chains results in CH₄ production. The increase in CH₄ generation by increasing protein degradability was also indicated by the positive relationship observed between NH₃-N concentrations and CH₄ production in the *in vitro* experiments with protected proteins.
3. The treatment of sunflower seed and sunflower meal with a malic acid solution plus heat (150°C) for 1 hour was effective to protect the sunflower protein (highly degradable and rich in sulphur-containing amino acids and tryptophan) from ruminal degradation. This treatment also decreased the production of methane and the CH₄/total VFA ratio, and increased the proportion of propionate without negatively affecting total VFA production. The effectivity of the treatment was higher in the sunflower seed than in the sunflower meal.
4. The overprotection of sunflower seed and sunflower meal by excessive heat duration (150°C for 3 h) should be avoided because it reduced the fermentability of both feeds and led to protein damage, as indicated by the increased NDIN and ADIN fractions, as well as by the reduced values of fermentation parameters.
5. Although using the same dose of malic acid used in the protective treatment or its disodium salt as additives presented some advantages in the efficiency of fermentation of sunflower seed and sunflower meal, the effects were markedly lower than those obtained with the protective treatment.
6. The variation in the positive effects of the treatment of sunflower seed and sunflower meal with malic acid solution plus heat (150°C for 1 hour) observed by changing the

donors' diet may be associated with the degradative activity -especially proteolytic activity- of the microbial inoculum, but also with differences in the amount of endogenous degradable protein added with the inoculum.

CHAPTER 8.-

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8. REFERENCES

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