UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,

ALIMENTARIA Y DE BIOSISTEMAS

CONTROL OF PROTEIN DEGRADATION IN THE RUMEN FOR IMPROVING PROTEIN EFFICIENCY AND REDUCING POLLUTING EMISSIONS

PhD THESIS

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M.Sc. in Animal Production and Health

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DISCLAIMER

Some results of this PhD. Thesis are already published:

- Experiment 1: Haro, A.N., Carro, M.D., de Evan, T., González, J. 2018. Protecting protein against ruminal degradation could contribute to reduced methane production. *Journal of Animal Physiology and Animal Nutrition* 102(6): 1482-1487. https://doi.org/10.1111/jpn.12973
- Experiment 2: Haro, A.N, González, J., de Evan, T., de la Fuente, J., Carro, M.D. 2019. Effects of feeding rumen-protected sunflower seed and meal protein on feed intake, diet digestibility, ruminal and cecal fermentation, and growth performance of lambs. *Animals* 9(7): 415. https://doi.org/10.3390/ani9070415

The rest of results will be submitted for publication to:

- Experiment 3: Haro et al. Carcass characteristics and meat composition and fatty acid profile in lambs fed sunflower protein protected against rumen degradation. *Animals.*
- Experiment 4: Haro et al. Influence of feeding sunflower seed and meal protected against ruminal fermentation on ruminal fermentation, bacterial composition and *in situ* degradability in sheep. *Animal Feed and Technology*.

ABBREVIATIONS / ABREVIATURAS

Abbreviation / Abreviatura	English	Castellano
*a**	Redness index	Índice de enrojecimiento
ADF	Acid detergent fiber	Fibra ácido detergente
ADIN	Acid detergent insoluble N	Detergente ácido insoluble N
ADL	Acid detergent lignin	Lignina ácido detergente
AGPR	The average gas production rate	Ritmo medio de producción de gas
ATP	Adenosine triphosphate	Trifosfato de adenosina
*b**	Yellowness index	Índice de amarillez
BUW	Buttock width	Ancho de cadera
C*	Chromaticity	Cromaticidad
c	Fractional rate of gas production	Ritmo fraccional de producción de gas
CON	Control diet	Dieta control
CTD	Thoracic depth	Profundidad torácica
CP	Crude Protein	Proteína Bruta
CWD	Carcass width	Ancho de la canal
DDGS	Distillers dried grains and soluble	Granos de destilería desecados con solubles
DMED	Dry matter effective degradability	Degradabilidad efectiva de la materia seca
DNA	Deoxyribonucleic acid	Ácido desoxirribonucleico

DNFB	Dinitrofluorobenzene	Dinitrofluorobenceno
DM / MS	Dry Matter	Materia Seca
ED / DE	Effective degradability	Degradación efectiva
EE	Ether extract	Extracto etéreo
EDTA	Ethylenediamine tetraacetic acid	Ácido etilendiaminotetraacético
EU	European Union	Unión Europea
FA	Fatty acid	Ácido graso
FAME	Fatty acid methyl esters	Ésteres metílicos de ácidos grasos
h*	Hue	Tono
HLL	Hind limb length	Ancho del miembro anterior
ICL	Internal carcass length	Longitud interna de la canal
k_c	Rate of particle comminution and mixing	Tasa de conminución y mezcla de partículas
k_d	Fractional degradation rate of the insoluble degradable fraction	Tasa de degradación fraccional de la fracción degradable insoluble
KKCF	kidney knob and channel fat	Grasa renal
k_p	The particle rumen outflow rate	Velocidad de salida del rumen de las partículas
*L**	Lightness index	Índice de luminosidad
LAB	Liquid-associated bacteria	Bacterias adheridas al líquido
lag	The time before starting gas production	El tiempo hasta que comienza la producción de gas

MAH	Malic acid and heading	Tratamiento con ácido málico y calor
M	Determination the value of the flattened meat	Determinación del valor de la carne aplanada
MUFA	Monounsaturated fatty acids	Ácidos grasos monoinsaturados
NDF / FND	Neutral detergent fiber	Fibra neutro detergente
NNP	Non-protein Nitrogen	Nitrógeno no proteico
OM / MO	Organic matter	Materia orgánica
OMF	Organic matter fermented	Materia orgánica fermentable
OMAF	Organic matter apparently fermented	Materia orgánica aparentemente fermentable
PGP	The asymptotic gas production	La producción asintótica de gas
PUFA	Polyunsaturated fatty acid	Ácidos grasos poliinsaturados
RUP	Undegraded protein in the rumen	Proteína del alimento no degradada en el rumen
RDP	Degradable protein in the rumen	Proteína degradable en el rumen
SAB	Solid-associated bacteria	Bacterias adheridas al solido
SFA	Saturated fatty acids	Ácidos grasos saturados
SM	Sunflower meal	Harina de girasol
SSM	Sunflower seed-meal mixture	Mezcla de semilla y harina de girasol
SS	Sunflower seed	Semilla de girasol

SAS	Statistical analysis system	Sistema de análisis estadístico
T	External area left by the eject water	Área externa dejada por el agua
TR	Treatment	Tratamiento
VFA / AGV	Volatile fatty acid	Ácido graso volátil

SUMMARY

Ruminants have the capacity of using fibrous feeds and low quality proteins to produce high-quality foods for humans, but they also have a low efficiency of nitrogen (N) utilization that is usually below 30%. As a consequence, a large part of the dietary N is excreted to the environment and contributes to soil and water eutrophication, being this problem especially marked in high-producing ruminants. Rumen metabolism is the most important factor contributing to the inefficient use of N in ruminants, especially in situations of an imbalance between the NH_3-N generated in the degradation of protein and the use of NH_3-N by the microbiota for microbial protein synthesis. In addition, it has been shown that protein degradation in the rumen generates CH_4, thus contributing to greenhouse gases emissions. Therefore, reducing the ruminal degradation of protein cannot only increase the amount of feed protein reaching the small intestine, but also can reduce polluting emissions such as N and CH_4. The general objective of this Doctoral Thesis is to investigate some aspects of the protein degradation in the rumen that are directly related to N losses and CH_4 production, and in order to achieve this objective four Experiments (two *in vitro* and two *in vivo*) were conducted.

The objective of Experiment 1 was to evaluate the inclusion of sunflower seed (SS) and sunflower meal (SM) protected against ruminal degradation in high-cereal diets on *in vitro* ruminal fermentation and CH_4 production. Samples of SS and SM were sprayed with a solution of malic acid 1 M (400 ml/kg sample) and dried at 150°C for 1 h as a protective treatment. Four diets were formulated to contain either 13 (low) or 17 (high) g of crude protein (CP)/100 g dry matter (DM), and included SS and SM either untreated (13CON and 17CON diets) or treated as before described (13TR and 17TR diets). Diets were incubated *in vitro* with rumen fluid from sheep for 8 and 24 h. The treatment did not affect ($p \geq 0.57$) total volatile fatty acid (VFA) production at any

incubation time, but it reduced ($p < 0.05$) NH_3-N concentrations by 19.2 and 12.5% at 8 and 24 h respectively. Both CH_4 production and CH_4/VFA ratio were lower ($p < 0.02$) in TR than in CON diets at 8 h, but differences disappeared ($p > 0.05$) at 24 h. The treatment increased the molar proportion of propionate ($p = 0.001$) and reduced that of isovalerate ($p = 0.03$) at 8 h compared with CON diets, but only a reduction of isovalerate proportion ($p = 0.03$) was detected at 24 h. There were no treatment x crude protein level interactions ($p > 0.05$) in any parameter, but high-protein diets had greater NH_3-N concentrations ($p < 0.001$) and lower VFA production ($p < 0.001$) than low-protein diets at 24 h. The treatment reduced protein degradation, and CH_4 production was decreased by 4.6 and 10.8% for low-and high-protein diets, respectively, at short incubation times without affecting VFA production, thus improving fermentation efficiency and decreasing polluting emissions.

The objectives of Experiments 2 and 3 were to analyze the efficacy of a treatment combining malic acid and heating (MAH) to protect the protein in SS and SM against rumen degradation and to improve the growth, carcass characteristics and meat quality of lambs. Two high-cereal concentrates, either including untreated SS and SM (control concentrate) or MAH-treated SS and SM (MAH concentrate), were formulated. The Experiment 2 was conducted to analyze the 12-h *in vitro* fermentation of untreated and MAH-treated SS and SM samples, as well as of both concentrates, using ruminal fluid from sheep as inoculum. The results indicated that the MAH treatment modified the rumen fermentation pattern of SS, SM and the concentrate, increasing molar proportions of propionate. In addition, MAH treatment reduced ($p = 0.009$) the NH_3-N concentrations for SM and tended ($p = 0.065$) to reduce them for the concentrate. However, no effects ($p \geq 0.100$) on CH_4 production were observed for any incubated feed. In Experiment 3, two homogeneous groups of 12 Lacaune lambs each (14.2 ± 0.35

Kg body weight) were fed either the control or the MAH concentrate. Lambs were fed concentrate and barley straw *ad libitum* for 40 days (until reaching about 26 kg body weight). Feed intake and growth of lambs were recorded, blood samples were taken on days 0, 20, and the slaughter day for analysis of urea-N and amino acid-N, diet digestibility was determined, and ruminal and cecal samples were collected after slaughter. In addition, carcass characteristics and quality and fatty acid profile of meat were assessed. There were no significant effects of the MAH treatment on feed intake and growth of lambs. Organic matter digestibility tended to be greater ($p = 0.07$) in MAH-fed lambs than in the control group, but there were no differences ($p \geq 0.33$) in the digestibility of CP, neutral detergent fiber (NDF) and acid detergent fiber (ADF). The hot carcasses of the lambs fed the MAH-treated concentrate were 7.9% heavier than those of the control group, but differences did not reach the significance level ($p = 0.212$). No differences between groups were detected in plasma concentrations of urea-N and amino acid-N ($p = 0.755$ and 0.500, respectively). There were no effects ($p \geq 0.172$) of MAH treatment on ruminal papillae characteristics and post-mortem VFA concentrations and profile in the rumen and the cecum. The color of the ruminal epithelium was darker ($p = 0.003$) in the lambs fed the MAH-concentrate compared with the control group, which was attributed to either to a corrosive action of malic acid or to a greater abrasion of the MAH-treated sunflower husks.

Compared with control lambs, those fed the MAH-concentrate showed greater ($p = 0.016$) amount of dorsal fat and greater ($p \leq 0.016$) values of the color parameters *a** (redness) and C* (chromaticity) in the *rectus abdominis* muscle. However, there were no differences ($p > 0.05$) in carcass measurements and in water-holding capacity, chemical composition, pH or color of *longissimus dorsi* muscle. Fatty acid profile of *longissimus dorsi* muscle was not affected ($p > 0.117$) by feeding the MAH-treated

concentrate, with the exception of a trend ($p = 0.055$) to greater concentrations of C14:0 in the MAH-fed lambs. In conclusion, the MAH treatment increased the *in vitro* fermentation of SS, reduced the *in vitro* protein degradability of SM, and modified the VFA profile towards greater propionate production. However, under the conditions of the present study the inclusion of MAH-treated SS and SM in a concentrate for growing lambs did not influenced significantly feed intake, diet digestibility, growth performance and meat fatty acid profile, although it increased hot carcass weight by 7.9% and the amount of dorsal fat.

In Experiment 4, the effects of spraying a solution of malic acid (1 M; 400 ml/kg) and ulterior heating to protect against ruminal degradation the proteins of SS and SM, as well as of a mixture of these both feeds (SSM; 45:55) were studied using *in vivo*, *in situ* and *in vitro* methods. Four rumen-fistulated sheep were fed two mixed diets composed of oat hay and concentrate (40:60) and differing only in the concentrate, that was either the control or the MAH concentrate used in Experiments 2 and 3. Sheep were fed the diets at 40 g DM/kg body weight$^{0.75}$ into six equal meals per day using automatic dispensers. A crossover design with two 24-day experimental periods was used, and each period included successively 10 days of diet adaptation, 9 days for performing *in situ* incubations of SS, SM and SSM, one day for measuring ruminal parameters, and two days for rumen empting. From day 6 onwards a solution of $(^{15}NH_4)_2SO_4$ was continuously infused into the rumen of each sheep to label ruminal bacteria. Feeding the MAH diet did not affect either ruminal pH or concentrations of NH_3-N and total VFA, but decreased ($p \leq 0.009$) molar proportions of acetate and propionate and increased those of butyrate ($p < 0.001$). In addition, organic matter (OM) and lipid contents in ruminal bacteria were lower in sheep fed the MAH diet compared with the control diet, whereas both N content and ^{15}N enrichment were greater ($p \leq$

0.037). Estimates of effective degradability (ED) of different feed fractions in SS, SM and SSM were obtained considering the ruminal rates of particle comminution and passage and correcting the values for the microbial contamination measured by using the ^{15}N infusion technique. The MAH treatment decreased the ED of most fractions in all tested feeds, increasing the supply of by-pass crude protein (CP) by 19.1 and 120% for SS and SM, respectively, and by 34% for crude fat in SS. The MAH treatment also increased the *in vitro* intestinal digestibility of the by-pass CP both for SS (from 60.1 to 75.4%) and SM (from 83.2 to 91.0%). The simultaneous heating of both feeds performed in SSM reinforced the protective effect increasing the by-pass CP without altering its intestinal digestibility. As a result, the intestinal digested CP content in SSM increased by 15.6% compared with the value estimated from the results obtained for SS and SM incubated independently. The results also confirm the previous observation that the effectiveness of MAH treatment is greater for high-CP feeds.

RESUMEN

Los rumiantes tienen la capacidad de utilizar alimentos fibrosos y proteínas de baja calidad para producir alimentos de valor nutritivo para los humanos, pero también tienen una baja eficiencia de utilización del nitrógeno (N), que generalmente es inferior al 30%. Como consecuencia, una gran parte del N de la dieta es excretada al medio ambiente y contribuye a la eutrofización del suelo y el agua, siendo este problema especialmente marcado en los rumiantes de alta producción. El metabolismo ruminal es el factor más importante que determina el uso ineficiente de N en rumiantes, especialmente en situaciones de desequilibrio entre el NH_3-N generado en la degradación proteica y la captación de NH_3-N por los microorganismos ruminales para la síntesis proteica. Además, se ha demostrado que la degradación de las proteínas en el rumen genera CH_4, lo que contribuye a las emisiones de gases de efecto invernadero. Por tanto, reducir la degradación ruminal de las proteínas no solo puede aumentar la cantidad de proteína del alimento que llega al intestino delgado, sino que también puede reducir las emisiones contaminantes, como son el N y el CH_4. El objetivo general de esta Tesis Doctoral es investigar algunos aspectos de la degradación proteica en el rumen que están directamente relacionados con las pérdidas de N y la producción de CH_4. Para lograr este objetivo se llevaron a cabo cuatro experimentos, dos *in vitro* y dos *in vivo*.

El objetivo del Experimento 1, fue evaluar la inclusión de semillas de girasol (SS) y harina de girasol (SM), protegidas frente a la degradación ruminal, en dietas con alto contenido en cereales sobre la fermentación ruminal *in vitro* y producción de CH_4. Para el tratamiento de protección (TR) se pulverizaron muestras de SS y SM con una solución de ácido málico 1 M (400 ml/kg de muestra) y se secaron a 150°C durante 1 h. Se formularon cuatro dietas para contener 13 (nivel bajo) o 17 (nivel alto) g de proteína

bruta (PB)/100 g de materia seca (MS), y se incluyeron SS y SM sin tratar (dietas: 13CON y 17CON) o tratadas como se ha descrito anteriormente (dietas: 13TR y 17TR). Las dietas se incubaron *in vitro* con líquido ruminal de ovejas durante 8 y 24 h. El tratamiento no afectó ($p \geq 0,57$) la producción total de ácidos grasos volátiles (AGV) a ningún tiempo de incubación, pero redujo ($p < 0,05$) las concentraciones de NH_3-N en 19,2 y 12,5% a las 8 y 24 h, respectivamente. Tanto la producción de CH_4 como la relación CH_4/AGV fueron menores ($p < 0,02$) para las dietas tratadas que para las dietas CON a las 8 h, pero las diferencias desaparecieron ($p > 0,05$) a las 24 h. El tratamiento aumentó la proporción molar de propionato ($p = 0,001$) y redujo la de isovalerato ($p = 0,03$) a las 8 h en comparación con las dietas CON, pero solo se detectó una reducción de la proporción de isovalerato ($p = 0,03$) a las 24 h. No hubo interacciones tratamiento x nivel de proteína bruta ($p > 0,05$) en ningún parámetro, pero las dietas altas en proteína originaron mayores concentraciones de NH_3-N ($p < 0,001$) y menor producción de AGV ($p < 0.001$) que las dietas bajas en proteína a las 24 h. En resumen, el tratamiento protector redujo la degradación de la proteína, y la producción de CH_4 (4,6 y 10,8% para las dietas bajas y altas en proteína, respectivamente) en tiempos de incubación cortos sin afectar la producción de AGV, mejorando así la eficiencia de fermentación y disminuyendo las emisiones contaminantes.

Los objetivos de los Experimentos 2 y 3 fueron analizar la eficacia de un tratamiento que combina ácido málico y aplicación de calor (MAH) para proteger la proteína en SS y SM contra la degradación del rumen, así como para mejorar el crecimiento, las características de la canal y la calidad de la carne de corderos en cebo. Se formularon dos concentrados basados en cereales, que incluían SS y SM sin tratar (concentrado control) o SS y SM tratados con MAH (concentrado MAH). El Experimento 2 consistió en analizar la fermentación *in vitro* de muestras SS y SM no

tratadas y tratadas con MAH, así como de ambos concentrados, usando fluido ruminal de oveja como inóculo e incubaciones de 12 h de duración. Los resultados indicaron que el tratamiento MAH modificó el patrón fermentativo de SS, SM y el concentrado, aumentando las proporciones molares de propionato. Además, el tratamiento MAH redujo ($p = 0,009$) las concentraciones de NH_3-N para SM y tendió ($p = 0,065$) a reducirlas para el concentrado. Sin embargo, no se observaron efectos ($p \geq 0,100$) en la producción de CH_4 para ningún alimento incubado.

En el Experimento 3, se usaron dos grupos homogéneos de 12 corderos Lacaune cada uno (14,2 ± 0,35 Kg de peso vivo) que fueron alimentados con el concentrado control o con el concentrado MAH. Los corderos recibieron concentrado y paja de cebada *ad libitum* durante 40 días, hasta alcanzar aproximadamente 26 kg de peso vivo. Se registraron semanalmente la ingesta de alimento y el crecimiento de los corderos, se tomaron muestras de sangre los días 0, 20 y el día del sacrificio para el análisis de urea-N y N aminoacídico, se determinó la digestibilidad de la dieta y se recogieron muestras ruminales y cecales después del sacrificio. Además, se evaluaron las características de la canal y la calidad y el perfil de ácidos grasos de la carne. No hubo efectos significativos del tratamiento MAH sobre la ingestión de alimento y el crecimiento de los corderos. Sin embargo, la digestibilidad de la materia orgánica tendió a ser mayor ($p = 0,07$) en los corderos alimentados con MAH que en el grupo control, pero no hubo diferencias ($p \geq 0,33$) en la digestibilidad de la PB, fibra neutro detergente (FND) y fibra ácido detergente (FAD). El peso de la canal caliente de los corderos alimentados con el concentrado MAH fue 7,9% mayor que el de las canales del grupo control, pero las diferencias no alcanzaron el nivel de significación estadística ($p = 0,212$). No se detectaron diferencias entre grupos en las concentraciones plasmáticas de urea-N y N aminoacídico ($p = 0,755$ y $0,500$, respectivamente), ni hubo efectos ($p \geq 0,172$) del

tratamiento MAH sobre las características de las papilas ruminales y las concentraciones y el perfil de AGV post-mortem en el rumen y el ciego. El color del epitelio ruminal fuer más oscuro ($p = 0,003$) en los corderos alimentados con el concentrado MAH que en el grupo control, lo que posiblemente fue debido a una acción corrosiva del ácido málico o a una mayor abrasión de las cáscaras de girasol tratadas con MAH.

En comparación con los corderos control, los corderos alimentados con el concentrado MAH tuvieron una mayor cantidad ($p = 0,016$) de grasa dorsal y mayores valores ($p \leq 0,016$) de los parámetros de color *a** (enrojecimiento) y C* (cromaticidad) en el músculo *rectus abdominis*. Sin embargo, no hubo diferencias ($p > 0,05$) en las medidas de la canal ni en la capacidad de retención de agua, composición química, pH y color del músculo *longissimus dorsi*. El perfil de ácidos grasos del músculo *longissimus dorsi* no se vio afectado ($p > 0,117$) por el concentrado administrado, con la excepción de una tendencia ($p = 0,055$) a mayores concentraciones de C14:0 en los corderos alimentados con el concentrado MAH. En conclusión, el tratamiento con ácido málico y calor aumentó la fermentación *in vitro* de SS, redujo la degradabilidad de la proteína *in vitro* de SM y modificó el perfil de AGV hacia una mayor producción de propionato. Sin embargo, bajo las condiciones del presente estudio, la inclusión de SS y SM tratados con MAH en un concentrado para corderos en cebo no influyó significativamente en la ingestión de alimento, la digestibilidad de la dieta, el rendimiento productivo y el perfil de ácidos grasos de la carne, aunque aumentó el peso de la canal caliente en un 7,9 % y la cantidad de grasa dorsal.

En el Experimento 4 se estudió, utilizando métodos *in vivo*, *in situ* e *in vitro*, la eficacia de tratar SS, SM y una mezcla de ambas (SSM; 45:55) con una solución de ácido málico (1 M; 400 ml/kg) y calor para proteger contra la degradación ruminal de la

proteína. Se usaron cuatro ovejas fistuladas en el rumen que fueron alimentadas con dos dietas mixtas compuestas de heno de avena y concentrado en proporción 40:60 y que diferían solo en el concentrado, que era el concentrado control o el concentrado MAH utilizado en los Experimentos 2 y 3. Las ovejas fueron alimentadas con dietas a 40 g MS/kg de peso metabólico^{0,75} repartidas en seis comidas iguales por día utilizando dispensadores automáticos. Se utilizó un diseño cruzado con dos períodos experimentales de 24 días, y cada período incluyó sucesivamente: 10 días de adaptación a la dieta, 9 días para realizar incubaciones *in situ* de SS, SM y SSM, un día para medir los parámetros ruminales y dos días para el vaciado ruminal. Desde el día 6 en adelante, se infundió continuamente una solución de $(^{15}NH_4)_2SO_4$ en el rumen de cada oveja para marcar las bacterias ruminales. La administración de la dieta MAH no afectó ni al pH ruminal ni a las concentraciones de NH_3-N y AGV totales, pero disminuyó ($p \leq 0,009$) las proporciones molares de acetato y propionato y aumentó las de butirato ($p < 0,001$). Además, el contenido de materia orgánica (MO) y lípidos en las bacterias ruminales fue menor en las ovejas alimentadas con la dieta MAH en comparación con la dieta control, mientras que su contenido de N y el enriquecimiento en ^{15}N fueron mayores ($p \leq 0,037$). En este trabajo se estimó la degradabilidad efectiva (DE) de las diferentes fracciones químicas de SS, SM y SSM considerando las tasas ruminales de conminución y paso de partículas y se corrigieron los valores para la contaminación microbiana, que fue medida usando la técnica de infusión de ^{15}N. El tratamiento con MAH disminuyó la DE de la mayoría de las fracciones en todos los alimentos incubados, aumentando el aporte de PB by-pass en 19,1 y 120% para SS y SM, respectivamente, y en 34% para la grasa en SS. El tratamiento con MAH también aumentó la digestibilidad intestinal *in vitro* de la PB by-pass tanto para SS (de 60,1 a 75,4%) como para SM (de 83,2 a 91,0%). El tratamiento simultáneo de ambas muestras realizado en SSM reforzó el efecto protector

de MAH, aumentando la PB by-pass sin alterar su digestibilidad intestinal. Como consecuencia, el contenido en PB digerida en el intestino en la mezcla SSM aumentó en un 15,6% en comparación con el valor estimado a partir de los resultados obtenidos para SS y SM cuando se incubaron de forma independiente. Los resultados también confirman la observación previa de que la efectividad del tratamiento MAH es mayor para los alimentos con alto contenido en PB.

I. INTRODUCTION AND OBJECTIVES

Ruminants can convert fibrous feeds and low quality proteins into nutrients that allow them to produce high-quality feeds for humans. In addition, they have the availability to use non-protein nitrogen (NNP) compounds in their diet due to the symbiotic relationship with ruminal microorganisms, which also provide them all B vitamins (Mackie, 1997). However, as a consequence of this adaptation they have a low efficiency of nitrogen (N) utilization, which is usually less than 30% of total N intake. This low efficiency is partly attributed to the high ruminal degradation of most vegetable proteins (non-protected proteins), which is associated to an excessive ammonia production in the rumen that cannot be used by ruminal microorganisms. The excess of ammonia has to be transformed in urea in a process that represents an energy cost for the host animal. Moreover, a great fraction of the dietary amino acids is transformed by ruminal microorganisms into compounds (D-amino acids, amino sugars of bacterial cell walls, microbial nucleic acids, ...) that cannot be used for protein synthesis by the host animal. All this leads to an increase in environmental N excretions, which contribute to environmental pollution and can cause soil and water eutrophication. Because a great part of the N emissions are due to ammonia losses from the rumen, as results of an excess of rumen degradable protein, the control of protein degradation in the rumen could reduce the amount of N excreted into the environment.

Ruminal fermentation of feeds also generates carbon dioxide (CO_2) and methane (CH_4) that are released to the environment, contributing to the global greenhouse gases. Dietary characteristics are one of the most important factors influencing CH_4 production, and it is well known that diets rich in structural carbohydrates yield higher CH_4 emissions compared with those high in non-structural carbohydrates (Kumar et al., 2014). Although the fermentation of carbohydrates is the main source of CH_4 in the

rumen, a recent study by our group (Vanegas et al., 2017a) showed that pure protein fermentation also generates CH_4, and that the response in CH_4 production to protein supply differed with the basal substrate. In addition, the protection of sunflower protein against rumen degradation by a malic acid-heat treatment resulted in a reduction of *in vitro* CH_4 production that was attributed to reduced protein degradation (Vanegas et al., 2017b,c). The CH_4 generated from protein degradation is presumably due to the hydrogen generation in the fermentation of carbon skeletons resulting from amino acid deamination.

The concern on livestock production emissions and their influence on climate change has markedly increased in recent years. Livestock production generates CO_2 with an atmospheric lifetime of between 50 and 200 years, CH_4 with a time of permanence in the atmosphere of 12 years, and nitrous oxide (N_2O) with an atmospheric lifetime of 100 to 150 years and responsible for a sharp increase in the radiation modifying the climate (IPPC, 2014). According to the Spanish Ministry of Agriculture, Fishing and Food estimations (MAPA, 2019), agriculture in Spain produces approximately half of total CH_4 of anthropogenic origin, and about 97.9% of this amount is generated by livestock production. The CH_4 of livestock origin has two sources: 67.2% comes from feed digestive fermentation (enteric methane) and 32.8% originates from manure fermentation. The greatest amount (94.0%) of enteric methane is produced by ruminants, whereas pig production is the main contributor (72.9%) to the CH_4 generated from manure (MAPA, 2019). In addition, the CH_4 produced in ruminal fermentation constitutes a loss of energy for the host animal, ranging between 2 and 12% of the gross energy consumed by the animal (Gerber et al., 2013).

The manipulation of ruminant diets is a feasible alternative to reduce polluting emissions that should be further investigated, but without reducing the use of feeds that

cannot be utilized in the feeding of other animal species and humans (i.e. structural carbohydrates or NPN compounds).

The objective of this PhD. Thesis was to investigate some aspects of the protein degradation in the rumen that are related to an increase in N losses and CH_4 production. This general objective was divided in the following specific objectives:

1. Analyse the influence of including rumen-protected proteins in high-cereal diets on *in vitro* ruminal fermentation and CH_4 production.
2. Assess the effects of feeding sunflower proteins treated with malic acid and heat on productive performance, diet digestibility, rumen and cecal fermentation, carcass characteristics and meat quality and fatty acid profile in growing lambs.
3. Evaluate the efficacy of a combined malic acid-heat treatment applied to sunflower seeds and sunflower meal on ruminal fermentation, bacterial composition and *in situ* degradation of both feeds.

II. LITERATURE REVIEW

2.1. Rumen microbial fermentation

The small intestine is the main digestion site for non-ruminant animals, whereas in ruminants most of digestion takes place in the reticulum-rumen. Whereas this has important advantages for the host animal, such as the digestion of fiber, the synthesis of microbial protein or the supply of some vitamins, it also has disadvantages as the low efficiency of N use. As the reticulo-rumen is located at the beginning of the gastrointestinal tract, ruminal fermentation is the first digestion site and only the fraction of feed not degraded in the rumen will flow to the small intestine. As a consequence, as ruminal fermentation increases, the concentration of indigestible components in the digesta that flows from out the rumen increases (González et al., 1999). In order to increase the efficiency of protein utilization and reduce polluting emissions, it is convenient to partially displace the digestion site to the intestine, but without affecting negatively the ruminal digestion. Among the different feed fractions, proteins, and especially those in high-quality protein feeds, are optimal targets for this change in the digestion site, as their denaturation and/or their condensation with other feed components can reduce their degradation rate and consequently increase the by-pass fraction.

Ruminants have a quick rate of feed ingestion and afterwards ruminate the feed (Figure 1), as the anatomy and physiology of their digestive tract is adapted to these processes, especially by having three forestomachs (reticulum, rumen and omasum) and the abomasum (Mackie, 1997). The rumen is the compartment with greater capacity in adult animals, and its inner wall is covered by rough papillae that allow the absorption of some fermentation compounds (Carro, 2016). Ruminal fermentation is carried out by bacteria, protozoa, fungi and methanogenic archaea, which are responsible for

degrading the feeds and hydrolyzing the released nutrients. As a result of this fermentative activity, ruminal microbiota produces volatile fatty acids (VFA), mainly acetate, propionate and butyrate. The absorbed VFA are the main source of energy for ruminants, as they can represent between 70 and 80% of the total energy obtained from the diet (Seal and Reynolds, 1993). In addition, heat, gases (CO_2 and CH_4) and NH_3-N are generated in the fermentation process.

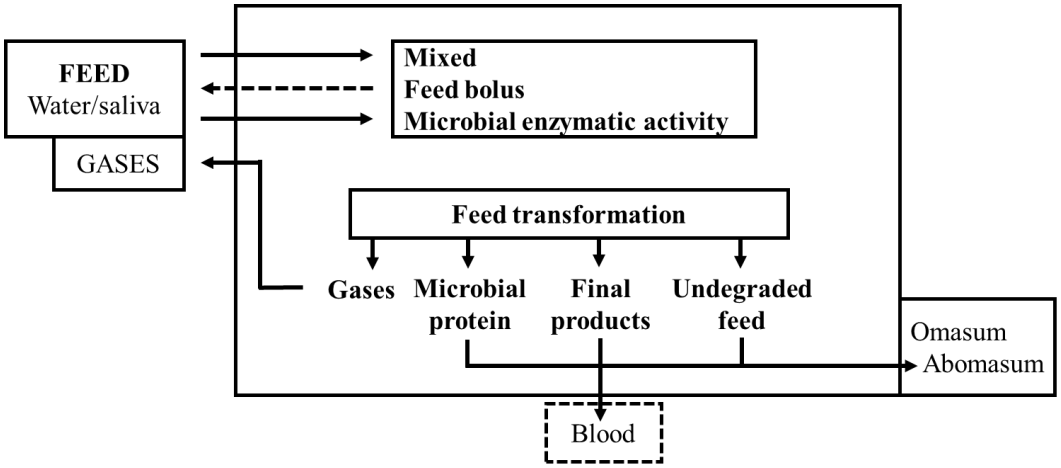

Figure 1. Transformation of feeds in the reticulum-rumen. Adapted from Van Lier and Regueiro (2008)

Due to the activity of the ruminal microbiota, ruminants can use fibrous components and NPN as part of their diet (Hofmann, 1993; Mackie, 1997). Other characteristics of the reticulum-rumen are the neutral or slightly acid pH of its content and the anaerobic conditions that provide an adequate environment for the maintenance and growth of the microbiota. Ruminal microorganisms degrade and ferment the fibrous components of the diet, synthesize high-quality microbial proteins and B vitamins, and can detoxify some toxic compounds of feeds. The reticulum has a mucosa that is characterized by a mucosal surface that forms folds (approximately 1 cm height) in the form of honeycomb, that enables the retention of foreign objects and plays an important role in the regurgitation of the bolus during rumination. The bolus is carried out to the mouth by reverse peristalsis. This process involves a wave of contraction followed by a

wave of relaxation, so as parts as the rumen are contracting, other sacs are dilating (Van Soest, 1994).

The mucosa of the omasum has also numerous and strong folds. This organ has no enzymatic function, but acts as a selective filter for solid particles, and is also a place for the absorption of water and minerals. Finally, the abomasum is a gastric compartment that has an acid pH and is equivalent to the stomach of the non-ruminants. The gastric juice produced by specialized cells in the mucosa of the abomasum is composed of hydrochloric acid, mucin, gastrin and digestive enzymes (Van Soest, 1994). The abomasum has two functional zones: the fundic region, that produces a large amount of secretions and has a pH close to 1.0, and the pyloric region, with a smaller and alkaline flow and a pH about 3.0. The endocrine system regulates the secretion abomasal. The distension of the pyloric zone, the increase in pH, and the concentration of VFA flowing to the abomasum increase the secretion of gastrin and hydrochloric acid (García and Gingins, 1969). Part of the VFA produced in the rumen flow to the abomasum and the small intestine mixed with the digesta. In addition, the structural carbohydrates not fermented in the rumen also flow out the rumen, can be fermented in the large intestine and the generated VFA can be absorbed. However, this VFA production is generally not quantitatively important compared to that generated in the rumen (Seal and Reynolds, 1993).

Ruminal microorganisms are wither strictly or facultative anaerobes, and are mainly constituted by bacteria, protozoa, fungi and methanogenic archaea. The bacterial populations are located in three different places in the rumen: adhered to the wall (epimural bacteria), closely associated with feed particles, and free floating in the ruminal fluid. The epimural bacteria hydrolyze urea very quickly, whereas the bacteria attached to feed particles (mainly cellulolytic and hemicellulolytic bacteria) ferment the

insoluble substrates. Finally, the bacteria floating in the rumen liquid ferment soluble and easily degradable substrates. The concentration of bacteria in the rumen is about 10^{10} - 10^{11} cells per ml of ruminal content, whereas the number of protozoa is lower (10^{5} - 10^{6} cells per ml). However, due to the greater size of protozoa compared to bacteria, they can contribute up to 50% of the microbial mass (Mackie, 1997; Stewart et al., 1997; Wright and Klieve, 2011).

Bacteria are classified according to the substrate that they ferment or their final fermentation products. The amylolytic bacteria predominate in the rumen of animals fed diets with high starch content, whereas the cellulolytic, hemicellulolytic and pectinolytic bacteria predominate in animals fed high-forage diets. The bacteria that use intermediate acids (lactate, succinate, malate, …) usually ferment the final products of the activity of other bacteria (Yokohama and Johnson, 1988). Lactate can be fermented to propionate, whereas succinate and malate can be converted to propionate and CO_2. Proteolytic bacteria produce a range of proteolytic enzymes (proteinases, exo and endopeptidases, deaminases, …) that degrade proteins to peptides and amino acids, whose deamination generates NH_3-N. The NH_3-N can also be rapidly generated from the hydrolysis of urea by the ureolytic bacteria. Lipolytic bacteria hydrolyze triglycerides and phospholipids, releasing glycerol and free fatty acids.

Ruminal protozoa are classified in two groups according to their morphology: flagellates and ciliates, the latter being the most abundant and important (Williams and Coleman, 1997). Protozoa can degrade a wide range of substrates and play a critical role in fiber degradation, but they can also store large amounts of starch decreasing the rate of VFA that causes a drop in ruminal pH in ruminants fed high-concentrate diets. Protozoa are also predators of bacteria, and therefore defaunation of a reduction of protozoa numbers usually increases the synthesis of microbial protein (Van Soest,

1994). The final products generated in protozoa metabolism are similar to those produced by ruminal bacteria, but generally they protozoa produce greater amounts of hydrogen, acetate and butyrate. Some methanogenic archaea live in close association with protozoa, thus having immediate access to the hydrogen generated in the fermentation, that is used to produce CH_4 (Williams and Coleman, 1997; Mackie et al., 2002).

The ruminal fungi are not essential for the life of ruminants, but they have an important role in the digestion of fiber, especially that found in low-quality and high-lignified forages (Van Soest, 1994). The number of fungi is increased when fibrous low-degraded feeds are fed, as they have low growth rate and require substrates with high retention time in the rumen. Fungi can account for up to 8% of the total microbial biomass of the rumen, and their number ranges from 10^3 to 10^6 cells per ml of ruminal content (Orpin, 1981; Akin and Rigsby, 1987).

Ruminal archaea are strictly anaerobes and produce CH_4 from CO_2 and hydrogen. The concentration of methanogenic archaea in the rumen has not been determined accurately, but they play a crucial role in ruminal fermentation by reducing hydrogen concentrations. Because the CH_4 emitted by ruminants contributes to global warming, its reduction is a major objective in the nutrition of ruminants (Hristov et al., 2013). Recent work with DNA sequencing has revealed that the most abundant phylum in the rumen is Euryarchaeota, which constitutes 99% of the ruminal archaea population. Ten genus of archaea have already been detected in the rumen, and the most abundant genus is Methanobrevibacter, which represents approximately 91% of total archaea (Zhou et al., 2017). The methanogenic are especially abundant in ruminants fed high-forage diets and the optimum conditions for their growth are similar to those for the cellulolytic bacteria (Yokohama and Johnson, 1988; Kim et al., 2010).

2.2. Protein degradation in the rumen

Ruminal microorganisms establish complex relationships among them, allowing the degradation of the feed ingested and consequently the use of their nutrients (Mackie, 1997). Feed proteins are degraded by microbial enzymes (proteases, peptidases and deaminases), which release peptides, amino acids and finally NH_3-N to the rumen contents. However, the intermediate metabolites generated are simultaneously utilized by the microorganisms to synthetize their own protein in the process known as microbial protein synthesis (Broderick et al., 1991).

The rate and extent of protein degradation in the rumen is affected by the type and concentration of enzymes produced, as well as by the number and type of peptide bonds that are susceptible to be broken (Carro, 2015). The secondary and tertiary structure of the proteins determines the accessibility of the microbial proteases to some peptide bonds, and therefore influences the degradability of the protein. Bacterial proteases are endo and exopeptidase enzymes bound to cells, but located on the cell surface to increase the interaction with the substrates. All factors affecting the number of ruminal microorganisms and their metabolic activity will influence the proteolytic activity (Ruiz and Ayala, 1987).

The synergic action of different types of proteases is necessary to complete the protein degradation, and therefore the participation of different ruminal microorganisms is required (Carro, 2015). It is estimated that up to 50% of ruminal bacteria have proteolytic activity on extracellular proteins and those with greater proteolytic activity are *Prevotella ruminicola, Ruminobacter amylophilus, Butirivibrio fibrisolvens* and *Streptococcus bovis*. Proteolytic activity in some bacteria such as *B. fibrisolvens* is extracellular, whereas in others such as *Prevotella ruminicola* and *Ruminobacter amylophilus* enzymes remain attached to cell membranes and quickly attack the proteins

existing in the ruminal contents (Wallace, 1999). The synergic action of different types of proteases liberates peptides and amino acids (Wallace et al., 1997). Protozoa play an important role in protein degradation because they engulf large feed particles and ruminal bacteria (Van Soest, 1994), although they cannot use NH_3-N for the novo synthesis of amino acids (Dijkstra, 1994).

Peptides are degraded to amino acids by the action of different peptidases, that can be extracellular or intracellular, requiring the incorporation of the peptides into the microbial cell if they are intracellular. Both peptides and amino acids can either be used directly for microbial protein synthesis or be degraded and deaminated to NH_3-N and carbon skeletons that can be used in the bacterial metabolism (Wallace et al., 1997, 1999). The use of the amino acids and peptides by the ruminal microbiota depends on the availability of energy in the rumen. If enough energy is available, they will be used for microbial protein synthesis, but if energy is not available they will be used in catabolic routes that produce ATP to meet the energy requirements of the microorganisms (Ørskov, 1992). The most important factors that affect the ruminal degradation of feed proteins are the type of protein, the interactions with other nutrients (mainly those supplying energy to ruminal microbiota), the predominant microbial population (which depends on the type of diet), the rate of passage of digesta through the rumen, and ruminal pH (Bach et al., 2005).

2.3. Synthesis of microbial protein

Dietary N compounds are partially degraded by ruminal microorganisms and used for the synthesis of microbial protein, and as a consequence the main final products of protein degradation are the feed protein undegraded in the rumen (RUP), the microbial protein, and NH_3-N (Bach et al., 2005). The RUP and the microbial protein leave the rumen and are further digested in the abomasum and small intestine, releasing

amino acids that are absorbed and transported in the blood circulation to body tissues (Dewhurst et al., 2000; Calsamiglia et al., 2010). The quantification of the synthesis of microbial protein is a crucial point in all protein feeding systems for ruminants, as it can represent between 40 and 90% of the amino acids reaching the small intestine (Bach et al., 2005). The microbial protein is synthesized (Figure 2) from the degradable fraction of the protein and the energy obtained from the degradation of carbohydrates (Schniffen and Robinson, 1987). It is important to notice that microbial protein synthesis and microbial protein flow are not equivalent, because part of the microbial protein synthesized in the rumen does not flow to the small intestine due to N recycling in the rumen, mainly as a consequence of the predation of bacteria by protozoa (Firkins, 1996).

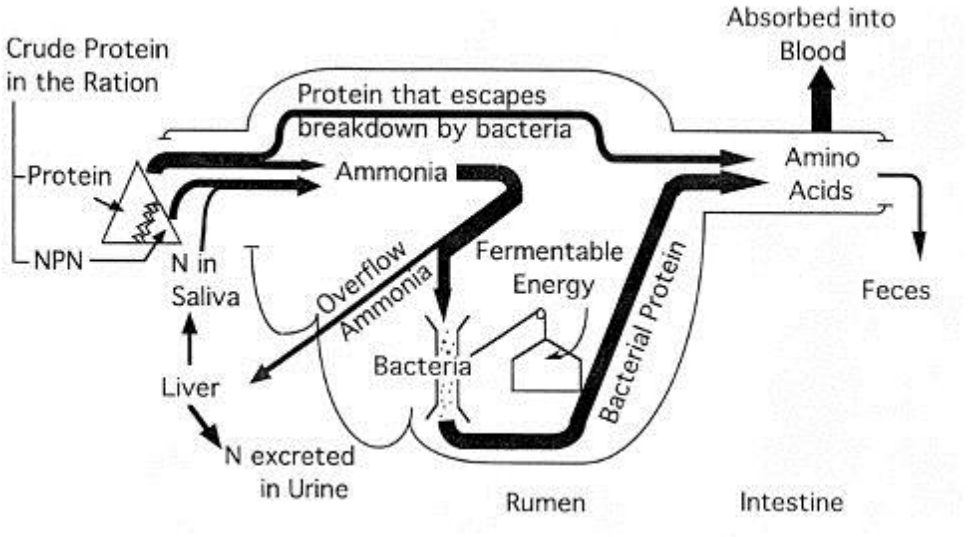

Figure 2. Ruminal degradation of nitrogen compounds and synthesis of microbial protein. Adapted from Satter and Roffler (1975)

Many factors influence the synthesis of microbial protein in the rumen, such as the energy available, the type of N, the synchronization between the supply of energy and N, protozoa activity, supplementation of unsaturated fats, passage rate of digesta, feeding level, and the presence of non-nutritive factors in the diet, among others

(Schniffen and Robinson, 1987; Bach et al., 2005). Dietary factors are the main contributors to the observed variability in the values of microbial protein synthesis reported in the literature, but the use of different experimental methodologies also contribute to this variability (Broderick and Merchen, 1992; Carro, 2001).

The amino acids that are absorbed in the duodenum of the host ruminant have three sources: the RUP, the endogenous protein (cellular desquamation and digestive juices), and the microbial protein synthesized in the rumen (Carro, 2015). Although microbial protein synthesis is a major factor in all protein feeding systems for ruminants (Clark et al., 1992; Firkins et al., 1996), its quantification is extremely complex in the practice, and it is usually estimated as the protein of microbial origin flowing to the abomasum or the duodenum. However, the recycling of microbial N in the rumen has been estimated to reach from 30 to 50% of the total microbial protein synthesized, although some authors have established wider ranges (20 - 90%) depending on the type of diet fed to the animals (Carro, 2015). Most of microbial N is of bacterial origin, because although protozoa represent a high proportion of the ruminal microbiota, its selective retention or sequestration in the rumen makes that its contribution to the duodenal flow of microbial protein is usually lower than 30% (Dijkstra et al., 1998; Reynal et al., 2003; Yáñez-Ruiz et al., 2006).

The values of the efficiency of synthesis of microbial protein reported in the literature are very variable, even in animals fed similar diets (Carro, 2015). Bach et al. (2005) and NRC (1996) reported that the average efficiency of the synthesis of microbial protein for diets based on forage, concentrates and mixtures of forage and concentrate, was 13.0, 13.2 and 17.6 g of microbial protein per 100 g of truly digested organic matter in the rumen, respectively. The availability of energy is a limiting factor

for microbial growth in the rumen, and therefore diets have to provide an adequate supply of energy and N, and if possible both should be synchronized in their release.

It has been observed that the synthesis of microbial protein is low when low quality forages are fed, due to the low and slowly degradation of carbohydrates. Microbial protein synthesis increases by feeding diets that contain a mixture of forage and concentrate, but it is markedly affected by the proportion of concentrate in the diet. It has been shown that diets with a high proportion of concentrates decrease the efficiency of microbial protein synthesis compared with those based on high-quality forages (Fébel and Fekete, 1996). The efficiency of microbial protein synthesis increases when easily fermentable carbohydrates constitute less than 30% of the total diet, but it decreases when the level of concentrate supplementation is greater than 70% (Fébel and Fekete, 1996). This decrease in the efficiency can be due to the rapid rate of non-structural carbohydrate degradation that causes a drop in rumen pH that affect negatively the microbial growth, especially that of cellulolytic bacteria, protozoa and archaea (Huber and Kung, 1981). When the pH drops, the microorganisms need to spend energy to keep a neutral pH inside the cells (Strobel and Russel, 1986).

Another limiting factor for microbial protein synthesis is the N availability. Bacteria can use NH_3-N, amino acids and peptides for the synthesis of microbial protein, but protozoa cannot use NH_3-N for the novo synthesis of amino acids (Jouany and Ushida, 1999). Ruminal microorganisms must have enough N to optimize the use of the energy generated in the fermentation of the organic matter, and a limitation of the amount of RDP in the diet can reduce the synthesis of microbial protein. However, an excess of RDP will cause an inefficient use of the protein, as well as energy losses for the host animal due to the need of transforming the excess of NH_3-N into urea (Carro, 2015).

2.4. Methane production: Influence of diet

Most of the enteric CH_4 produced by ruminants is generated in the rumen (85-95%) and only 5-15% of the total is produced in the large intestine (Carro et al., 2018). Methane is produced by the methanogenic archaea, and it is formed from the products resulting from the fermentation of the feed organic matter. The majority of archaea generates CH_4 from CO_2 and hydrogen ($CO_2 + 8\ H + \rightarrow CH_4 + 2\ H_2O$), being a minor proportion those that produce CH_4 by the reduction of acetic acid ($CH_3COOH \rightarrow CH_4 + CO_2$). Other minor precursors for CH_4 synthesis are the formic acid, methylamines, methanol, methanethiol and methyl sulfide.

The amount of enteric CH_4 generated daily by a ruminant depends on multiple factors, but among the dietary factors the amount of organic matter fermented, the type of macromolecules (starch, cellulose, proteins, ...), and the profile of the VFA produced in ruminal fermentation are the most important feed factors (Carro et al., 2018). The organic matter fermented consists mainly of carbohydrates and proteins, as fats fermentation in the rumen is limited to the fermentation of glycerol released from triglycerides (producing VFA) and the biohydrogenation of free unsaturated fatty acids. This process of biohydrogenation acts as a hydrogen sink, and therefore reduces the hydrogen available for CH_4 synthesis by the archaea. In addition, free fatty acids (mainly those unsaturated) have a toxic effect on archaea, protozoa and some bacterial species, especially fibrolytic bacteria, and therefore high levels of fat in the diet can reduce both CH_4 production and fiber digestibility. The negative action of free fatty acids is usually lower in animals fed high-concentrate diets which cause a low ruminal pH, compared with those fed high-forage diets.

The type of diet ingested by ruminants and the rate of fermentation are the main dietary factors influencing CH_4 production in the rumen. The fermentation of diets rich

in structural carbohydrates (high-forage diets) generates a greater amount of CH_4 per unit of feed intake than the fermentation of diets rich in nonstructural carbohydrates such as starch and sugars. Grazing animals usually have greater CH_4 emissions than animals raised under intensive conditions and receiving high-concentrate diets (Carro et al., 2018). The forage/concentrate ratio in the diet has a marked influence on the production of propionate, and it has been shown that increasing the proportion of concentrate up to 40% of the diet reduces slightly the generation of CH_4, but there is a marked reduction when concentrate proportion increases from 40 to 90%, a situation in which CH_4 can account for only 3% of the gross energy intake (Carro et al., 2018). The increase of the proportion of concentrate in the diet usually causes a decrease in ruminal pH due to the increase in the production of VFA and lactate, which causes a reduction of the cellulolytic and an increase of the amylolitic microbiota (Moss et al., 2000; Beauchemin et al., 2009; Martin et al., 2010). Under *in vitro* conditions (Van Kessel and Russell, 1996; Lana et al., 1998) it has been observed that ruminal pH values below 6.5 drastically reduce CH_4 production. However, a low correlation between CH_4 emission and ruminal pH has been reported in beef cattle ($r = 0.27$, $P < 0.05$), and therefore it has been suggested that there are mechanisms that allow methanogenic archaea to overcome episodes of low ruminal pH values, such as changes in methanogenic populations to species with greater tolerance to low pH values or its sequestration in protective microenvironments as biofilms or protozoa (Hünerberg et al., 2015).

Methane production has been usually related to the fermentation of carbohydrates, mainly those structural, whereas its generation from the fermentation of other substrates, such as protein, has received little attention (Carro and Miller, 1999; Vanegas et al., 2017a). The amount of protein degraded in the rumen is affected by the proteolytic activity of ruminal bacteria, the physical access of bacteria to the protein,

and the digesta retention time in the rumen, among other factors (Broderick et al., 1991; NRC, 2001). The proteolytic activity is closely related to the concentration of microorganisms in the rumen, and therefore to the availability of energy, N and other nutrients that affect microbial growth (Huhtanen and Hristov, 2009). When the degradation rate of the protein exceeds that of carbohydrates, large amounts of N are lost in the form of NH_3-N, causing a decrease in proteolytic activity (Nocek and Russell, 1988). In addition, the fermentation of the carbon chains resulting from the deamination of amino acids can also contribute to the supply of hydrogen to the methanogenic archaea, and therefore the degradation of the protein influences the production of CH_4 (Carro and Miller, 1999; Carro et al., 1999). Decreasing deamination by limiting the supply of RDP in the diet to the amount required for optimal microbial growth would be an effective method to reduce CH_4 production. Most unprocessed protein feeds contain a high proportion of RDP, but this fraction is lower in feeds that have been treated to protect the protein against ruminal degradation or that have been subjected to industrial treatments with a strong thermal impact. *In vitro* studies have shown that including the diet protein feeds protected against ruminal degradation reduces the proportion of RDP and increases the proportion of by-pass protein, contributing to lower CH_4 emissions (Vanegas et al., 2017b).

The inclusion of unsaturated fats in the diet of ruminants reduces the production of CH_4 by different mechanisms, such as the biohydrogenation of fatty acids, the increase in the production of propionate, and the inhibition of protozoa activity. It has been shown that the dietary supplementation of long-chain polyunsaturated fatty acids decreases the methanogenesis because these fatty acids are used as a hydrogen sink (Beauchemin et al., 2009). However, this practice can also cause negative effects on ruminal fermentation due to their toxic effect on some ruminal microorganisms, and

usually decreases fiber digestibility, feed intake and animal productivity (i.e., decrease in milk fat and/or protein content). Grainger and Beauchemin (2011) analyzed 27 scientific studies using diets containing less than 8% fat and observed that an increase of 10 g of fat per kg of diet caused a reduction in CH_4 production of 1.0 and 2.6 g per kg of DM intake in cattle and sheep, respectively. Supplementing the diet with fat with the only aim of reducing CH_4 production is a questionable strategy, but feeding high-fat byproducts (i.e. DDGS, olive cake) that do not increase feeding costs would be a feasible option (Hristov et al., 2013).

The use of feed additives with antimetanogenic potential has been extensively reviewed (Knapp et al., 2014; Kumar et al., 2014). Ionophore antibiotics have proven to have antimetanogenic effects, but these effects disappeared with time, and their use is banned in the European Union since 2006. Among the feed additives most investigated, there are some plant extracts such as tannins, garlic oil (it inhibits activity of methanogenic archaea), and saponins (inhibits the activity of protozoa), but they can also reduce fiber digestibility. Other additives such as organic acids (fumarate and malate) act as hydrogen sinks and have shown good *in vitro* results, but their *in vivo* effects are not consistent (Carro and Ungerfeld, 2015). Probiotics (yeasts and acetogens) have shown antimetanogenic activity only in some studies, and results from different studies are controversial (Knapp et al., 2014). In summary, there are currently no additives that can be used in practical feeding to effectively reduce CH_4 production without negatively affecting animal productivity.

2.5. Protein sources in ruminant feeding

Ruminants are able to use a great variety of N compounds due to the symbiotic relationship with ruminal microorganisms. Therefore, they can degrade from N compounds with large molecular weight and complex structure, such as animal proteins,

to simple compounds, such as urea and ammonium salts (Mejía and Mejía, 2007). Depending on its possible fermentation in the rumen, the CP of the diet is classified in two fractions: RDP and rumen-undegradable (RUP) or by-pass protein. Dietary protein formulation in ruminants is actually a double process, as the N requirements of the ruminal microbiota must be met in the form of RDP, whereas the amino acid requirements of the host animal are met by both the RUP and the microbial protein synthesized (Cochran et al., 1998; NRC, 2001).

Protein feeds can be either animal origin (meat meals, animal plasma, hydrolysates of intestinal mucosa) or plant origin. The by-products of soybean, sunflower and camelina, and peas are the most commonly used protein sources in Spain, due to their high protein content ranging from 20 to 51% of CP. The proportion of RDP in the in protein feeds can vary between 30 and 90%, although in most of feeds of plant origin the RDP is greater than 65% of total protein (FEDNA, 2018). Meals of animal origin are generally more resistant to degradation in the rumen than those of plant origin and have high protein content (45 - 85%), being also good sources of P and Ca. However, the use of meat and fish meal in ruminant feeding is banned in the European Union as a consequence of the bovine spongiform encephalopathy.

As shown in Figure 3, among the most commonly used protein concentrates, the corn gluten meal has the greatest protein content (60%), whereas the spring peas have the lowest content (20.6%). Sunflower meal has an intermediate content and, as average, contains between 28 to 36% protein. In addition, soybean meal contains between 44 to 48% protein, and is the most widely used protein feed in high-production ruminants due to the high quality of its protein. Protein degradability is high in cereal grains (75-80%), medium in soybean meal (65%), and high in spring peas and sunflower meal (85-90%). The RDP provides a mixture of peptides, free amino acids

and NH_3–N for the synthesis of microbial protein, which can account for 55 to 87% of total amino acid N flowing out the rumen (NRC, 2001; Clark et al., 1992).

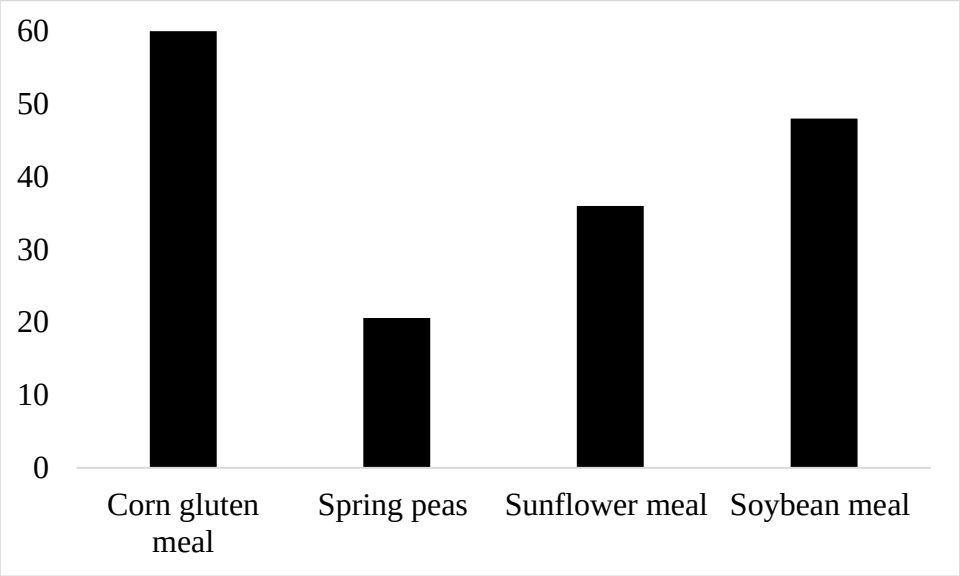

Figure 3. Average protein content (%) of some concentrate feeds commonly used in Spain: corn gluten meal, spring peas, sunflower meal and soybean meal (FEDNA, 2018)

It has been observed that the administration of true protein (soybean meal, sunflower, ...) is more effective in stimulating the intake and digestion of forage than the feeding of NPN (Köster et al., 1997; Cochran et al., 1998). The NPN compounds of the diet (i.e. urea and its derivatives, ammonium salts, …) are rapidly and completely degraded to NH_3-N in the rumen (Wallace et al., 1997), and therefore they must be used with caution as an excess can cause an intoxication by NH_3-N accumulation. It is recommended that urea supplementation does not exceed either 1% of the diet DM or the 25% of total N (FEDNA, 2018). In addition, the use of NPN compounds in the diet should be accompanied by a rapidly available energy source, so that they can be used by the ruminal microbiota. The excess of NH_3-N that cannot be used by the microorganisms has to be transformed into urea in the liver, and this process has an energy cost for the host animal (Köster et al., 1997; Cochran et al., 1998).

2.6. Production of sunflower in Spain

The production of sunflower, soybean and rapeseed accounts for about 80% of total world production of oilseeds, and the remaining 20% corresponds to cotton and peanuts, among others. Sunflower is one of the most commonly used protein feeds in Spain and Europe. About 800,000 ha of sunflower are cultivated in Spain, and this crop increased by 8% between 2010 and 2015 (MAPA, 2019). This crop has also increased in recent years, with a volume of production ranging from 800,000 to 1,000,000 t. Andalucía, Castilla y León and Castilla-La Mancha are the Autonomous Communities with the greatest production (MAPA, 2019).

Because of the high production in our country, sunflower is commonly used in ruminant feeding, although its production and price can fluctuate widely, thus affecting the cost of the diet (González et al., 1999). Sunflower is cultivated in Spain for oil production for human consumption, and it is calculated that about of 576,000 t of sunflower are obtained annually as a by-product, being mostly used in animal feeding (MAPA, 2019; FEDNA, 2018). Sunflower protein is rich in sulfur amino acids and tryptophan, but low in lysine, and therefore it amino acid profile balances well legume protein (FEDNA, 2018). However, the degradability of sunflower protein in the rumen is very high (above 80%), and protective treatments against ruminal degradation are required to increase the amount of by-pass protein (González et al., 1999).

2.7. Protection of protein against rumen degradation

The low nitrogen efficiency of ruminants is partially due to NH_3–N losses in the rumen, and therefore reducing ruminal degradability of protein would improve N efficiency and reduce environmental contamination, not only N but also CH_4 emissions as shown in different *in vitro* studies (Vanegas et al., 2017a,b). There are many physical and chemical methods to decrease the ruminal fermentation of proteins. These methods

can be physical (heat), chemical (application of chemical compounds) or a combination of both, and they modified the characteristics of the protein increasing its resistance to proteolytic enzymes (González et al., 1999).

Among the physical methods, the most widely used is heating, that alters the three-dimensional structure of the proteins without breaking the peptide bonds. This process involves the denaturation of the proteins, the reduction of both the solubility and the accessibility to microbial enzymes, with the consequent reduction of ruminal degradation (Blanchart, 1988). Furthermore, heating favors the condensation reactions between the aldehyde groups of the sugars and the free amino groups of the protein. However, excessive heating produces Maillard reactions that imply the formation of phenolic compounds and makes the protein indigestible (Broderick et al., 1991; Van Soest, 1994).

There are different chemical compunds that can be used to reduce the ruminal degradability of the protein, such as formaldehyde, acids, alcohols, tannins, etc. Formaldehyde has been widely used in the past, and causes a reduction in the solubility and degradation of the protein in the rumen as a consequence of its reaction with the amino groups. However, its use as a chemical treatment is currently banned in the EU (Tamminga, 1979; Walker, 2005).

Acids are chemical compounds that can be used to protect the protein, and its mode of action is based on the denaturation of the protein, even at a moderate low pH, by hindering the enzymatic attack of rumen microorganisms. Acids also exert an antimicrobial effect, thus reducing the microbial colonization of feeds. Among the acids that can be used with the purpose of protecting the protein against rumen degradation, malic and fumaric are of especial interest due to their effects as rumen modifiers (Nisbet and Martin, 1993). Numerous *in vitro* studies (reviewed by Carro and Ungerfeld, 2015)

that the addition of malate or fumarate as acids or their salts increase the production of propionate and may reduce CH_4 production. The main advantage for the use of malic acid in the treatment of protein protection is its high solubility in water, but its high cost and corrosion power are disadvantages (Díaz-Royón, 2016).

Any treatment must be effective in protecting the protein against ruminal degradation, but should not reduce either the intestinal digestibility of the protein, allowing the absorption of essential amino acids in the small intestine, or the microbial protein synthesis. The combination of physical and chemical treatments for protein protection has given positive results, in particular using malic acid and heat (Vanegas et al., 2017b). The application of combined acid-heat treatments has also economic advantages, as it can reduce the cost of heat treatment, allows the use of a lower acid dose, and reduces the possibility of generating irreversible Maillard reactions which cause overprotection of protein. However, overheating or the use of excessive chemical treatment can have detrimental effects on rumen degradation and/or on intestinal digestibility of protein. The level of protection depends not only on the type of acid used, but also on the dose, dilution, temperature and on heating time (Ouarti et al., 2006).

Arroyo et al. (2011) observed that the treatment of sunflower meal with malic acid at different concentrations and temperatures markedly increased the RUP content compared to untreated sunflower meal. Similarly, when treating sunflower meal with 2N solutions of either malic or orthophosphoric acid followed with heating at 150°C for 6 hr. Arroyo et al. (2013) observed that RUP content was increased by 26.7% regardless of the acid used (malic or orthophosphoric) and the intestinal digestibility of the protein increased by 20 and 11.8% for malic and orthophosphoric acid, respectively. Díaz-Royón et al. (2016) reported that a combined treatment of acid (malic or

orthophosphoric) and heating applied to sunflower meal and spring pea reduced their rumen degradation, shifting the digestion site to the small intestine. Vanegas et al. (2017a) also observed positive effects of a 1M malic acid-heat treatment of sunflower meal and seeds, indicating that this treatment was effective to protect the high degradable sunflower proteins. These results indicate that combined acid-heat treatments are effective in protecting the sunflower protein from ruminal degradation, but the effect may vary depending on the temperature applied and the duration of the heat treatment. In addition, the malic acid added in the treatment can also have a beneficial effect on ruminal fermentation as already discussed. However, the studies involving sunflower meal and seeds (Vanegas et al., 2017b,c) were conducted under *in vitro* conditions, and *in vivo* studies are necessary to confirm their results.

2.8. Meat sheep production in Spain

Sheep census in Europe is over 84 million heads, with Spain being the second country with the greatest census (19% of total in the EU) only after the United Kingdom which has almost 23 million (27% of total in the EU). There are currently 16.7 million heads of sheep in Spain (MAPA, 2019), of which 23% are located in Extremadura, followed by the Autonomous Communities of Castilla y León, Castilla la Mancha and Andalusia (17.7, 15.8 and 14.6% of total census, respectively). The rest of sheep population is distributed over the country (Table 1). Sheep census has declined sharply in the last years, as in 2007 reached 23 million heads (MAPA, 2019).

Table 1. Sheep population the Autonomous Communities of Spain (MAPA, 2019)

Autonomous Communities	Nº of animals	% of total
Andalucía	2,428,539	14.6
Aragón	1,701,793	10.3
Asturias	68,262	0.4
Baleares	282,906	1.7
Canarias	52,711	0.3
Cantabria	46,464	0.3
Castilla La Mancha	2,612,969	15.8
Castilla y León	2,930,949	17.7
Cataluña	534,288	3.2
Extremadura	3,810,655	23.0
Galicia	204,869	1.2
Madrid	107,533	0.6
Murcia	637,783	3.8
Navarra	471,162	2.8
País Vasco	297,224	1.8
La Rioja	111,856	0.7
Valencia	284,716	1.7
Total	16,584,679	100

In 2018, 118,568 tons of sheep meat were produced in Spain, with Castilla y León being the first producer of sheep meat (28,579 t and almost 4 million slaughtered heads), followed by Cataluña (17,733 t) and Castilla-La Mancha (16,496 t). The production of sheep meat has declined markedly in the last years, as reached 230,000 t between 2001 and 2004 (MAPA, 2019). A great part of the production (87,324 t in 2018) is exported to other countries, being France and Italy the main importers with 43.1% and 13.4% of exports, respectively.

Sheep are slaughtered at different stages of growth. The main types of lamb are "recental" lamb (25-26 kg of body weight and 15 kg of carcass as average), "lechazo" (10-11 kg of body weight), and "pascual" lamb (30 -35 kg of body weight and more

than 15 kg of carcass), representing 36, 24 and 22% of total slaughtered lambs, respectively (Sanz et al., 2008, MAPA, 2019). The production of heavy lambs, reaching 50 kg of body weight, has markedly increased in the last years, but this production is exported mainly to Arabian countries.

2.9. Diet influence on production and quality of lamb meat

Lamb diets must be formulated to meet the energy and protein requirements of animal tissues, but optimizing microbial protein synthesis in the rumen is a crucial point. Therefore, diets should provide balanced amounts of energy and N for ruminal microbiota (Dewhurst et al., 2000). Proteins are fundamental components in all animal tissues and are also necessary for the maintenance of vital functions, such as the renewal of tissues and lambs growth. The tissues of the animal can only use amino acids (either of microbial or feed origin) for protein synthesis, whereas main source of energy are the VFA generated in the ruminal fermentation of carbohydrates and the fatty acids, either absorbed in the small intestine or those from body fat mobilization (Seal and Reynolds, 1993).

Fattening lambs have a high growth rate and the microbial protein synthetized in the rumen cannot meet the metabolizable protein requirements of these animals; therefore, the use of by-pass protein can increase the productive performance and improve the quality of the meat (Díaz-Royón, 2016). Due to the role played by ruminal microorganisms in feed digestion and the high quality of the microbial protein, ruminant diets should be formulated to allow an optimal microbial growth, as a limitation in amino acids supply to the host animal can limit the productive performance and quality of sheep meat (Calsamiglia et al., 2010).

The nutritional management in sheep farms in Spain is variable. Due to the diversity of production systems (intensive, semi-intensive and extensive), the diets for

fattening lambs have wide ranges of protein content, which can vary between 15 and 20%. Even in the scientific studies conducted in studies with fattening lambs conducted in our country, great variations in protein content were observed. De Evan et al. (2019) and Pelegrin et al. (2019a) slaughtered lambs of Lacaune and Ripollesa lambs at 26 kg of body weight and provided diets with 17 and 20% protein, respectively. Martín et al. (2019) slaughtered Assaf lambs at 50 kg and fed diets with 16% protein. Pelegrin et al. (2019b) carried out a study to evaluate the effect of the inclusion of 17 and 20% of protein in the diet of Ripollesa lambs, and observed that the reduction of the protein level did not affect their productive performance. An excess of protein causes economic losses by increasing the cost of the diet, but also involves energy expenditure for the animal (due to urea synthesis), and increases environmental pollution without improving animal production (González et al., 1999, Díaz-Royón et al., 2016).

Lamb meat is considered a product of high nutritional value for humans, with high fat and protein content, although it varies with lamb age, slaughter weight and carcass type. The water content after slaughtering of lamb carcass is, as average, 65%, and losses during chilling can reach up to 5% in "lechazo" lambs (Aguilera, 2000; Aurousseau et al., 2004). Lamb fat is high in saturated fatty acids (SFA) due to the ruminal biohydrogenation of dietary fatty acids, which causes that ruminant products contain fats more saturated and with greater proportions of palmitic and stearic acids than that of non-ruminants. Lambs meat also contains high proportions of monounsaturated fatty acids (MUFA) such as oleic, which can reach concentrations up to 45% of total fatty acids (Bravo-Lamas et al., 2015), and lower content of polyunsaturated acids (PUFA) than the products of non-ruminant species. In ruminants, the PUFA ω-6 and ω-3 acids ingested in the diet are transformed into other MUFA and SFA fatty acids in the rumen by microbial biohydrogenation, and it is estimated that

only 10% of the fatty acid in the diet are incorporated into the tissues of ruminants (Nieto and Ros, 2012). In comparative studies, it has been observed that pigs have an intermediate PUFA/SFA ratio and a low ω-6 / ω-3 ratio compared with sheep (Díaz et al., 2005; Aguilera, 2000). Numerous studies have been conducted to analyze the effect of including different PUFA sources in the diet of lambs with the objective of modifying the fatty acid profile of meat. The success of this feeding strategy depends on the type of diet, the length of the feeding period, the PUFA source, and the protection of PUFA-source against rumen degradation (Pérez, 2010; Díaz-Royón et al., 2016).

III. MATERIAL AND METHODS

All procedures involving animals were conducted 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 European legislation (Directive 2010/63/UE), and experimental procedures were approved by the Animal Experimentation Ethics Committee of the Comunidad Autónoma de Madrid (Approval number PROEX 035/17).

3.1. Objective 1: Analyse the influence of including rumen-protected proteins in high-cereal diets on *in vitro* ruminal fermentation and CH_4 production

In order to reach this objective an *in vitro* experiment was conducted. Experiment 1. "Protecting protein against ruminal degradation could contribute to reduced methane production".

One sample of sunflower seeds (SS) and one of sunflower meal (SM) were obtained and ground (2 mm). Samples were weighed (500 g) and sprayed with a solution of 1 M malic acid at a rate of 400 ml per kg of substrate fresh matter, left at room temperature for 1 h and subsequently dried at 150°C for 1 h as a protective treatment (TR) against ruminal degradation (Vanegas et al., 2017a). Four total mixed diets were formulated according to a 2 × 2 fa ctorial arrangement of treatments. The diets had 13 (low) or 17 (high) g of crude protein (CP)/100 g dry matter (DM) and included SS and SM either untreated (13CON and 17CON diets) or treated as before described (13TR and 17TR diets). All feed ingredients were ground to pass a 1 mm screen and mixed. Ingredient and chemical composition of diets and chemical composition of SS and SM are shown in Table 2.

Four sheep (67.2 kg ± 2.31 kg body weight), each provided with a permanent rumen cannula, were used as rumen fluid donors for the *in vitro* incubations. Sheep were fed a mixed diet of grass hay and a commercial concentrate (1:1) at a daily rate of 42 g/kg body weight$^{0.75}$ distributed in two equal meals. Samples (300 mg DM) of each diet were accurately weighed into glass vials (115 ml). Ruminal contents from each sheep were obtained immediately before the morning feeding, strained through 4 layers of cheesecloth and the fluid mixed with a culture medium (1:4 ratio; vol/vol) at 39°C. The medium of Goering and Van Soest (1970) was changed by replacing the $(NH_4)HCO_3$ with $NaHCO_3$ and excluding the trypticase to obtain a N-free medium. Each vial was filled with 30 ml of the mixture under CO_2 flushing before being capped and incubated at 39°C. For each diet, a total of 16 vials were incubated, four with the ruminal fluid from each sheep (inoculum); therefore, there were four replicates per experimental treatment and sampling time.

After 8 h of incubation, gas production was determined in 2 vials per diet and inoculum using a pressure transducer (Wider eager Wide Range Pressure Meter; Sper Scientific LTD, Scottsdale, AZ, USA) and a plastic syringe. A gas sample (15 ml) was removed from each vial and stored in a vacuum airtight tube (Terumo Europe N.V., Leuven, Belgium) until CH_4 analysis by gas chromatography. Vials were uncapped, the pH was measured (Crison Basic 20 pHmeter, Crisson Instruments, Barcelona, Spain) and 5 ml of vials content were added to 5 ml of 0.5 M HCl and kept frozen (–20° C) until volatile fatty acid (VFA) and NH_3-N analysis. The rest of the vials were processed as before described after 24 h of incubation.

Table 2. Chemical composition and ingredients of experimental diets used as substrates for *in vitro* incubations and chemical composition of sunflower seed and sunflower meal either untreated (CON) or treated (TR) to protect the protein against ruminal degradation

Item	Experimental diets[a]				Sunflower seed[b]		Sunflower meal[b]	
	13CON	13TR	17CON	17TR	CON	TR	CON	TR
Chemical composition (g/kg dry matter)								
Organic matter[c]	961	960	952	951	969	970	927	932
Crude protein	131	130	170	169	179	171	359	337
Neutral detergent fibre	325	322	369	366	292	271	490	466
Acid detergent fibre	127	125	165	166	159	147	275	261
Acid detergent lignin	26.2	25.6	39.0	40.2	53.8	48.7	88.2	84.5
Ether extract	85.3	85.7	83.7	85.3	467	459	12.9	20.2
Non-structural carbohydrates[d]	420	422	329	331	31.0	69.0	65.1	108
Ingredients (g/kg fresh matter)								
Soft wheat grains[e]	469	464	349	329				
Maize grains	156	155	116	110				
Straw	150	150	150	150				
CON-sunflower seed	150	-	150	-				
TR-sunflower seed	-	153	-	153				
CON-sunflower meal	75.0	-	235	-				
TR-sunflower meal	-	79.6	-	259				

[a] 13CON, 13TR, 17CON and 17TR: Diets with two levels of crude protein (130 and 170 g/kg dry matter) and containing either untreated (CON) or treated (TR) sunflower seed and sunflower meal to protect protein against ruminal degradation. [b]Sunflower seed and meal were either untreated (CON) or treated with a solution 1 M malic acid (400 ml/kg) and dried at 150°C for 1 h to protect protein against ruminal degradation (TR). [c]Calculated as 1000–ash. [d]Calculated as 1000 – (ash + crude protein + neutral detergent fibre + ether extract). [e]A wheat variety with greater starch and lower protein thanhard wheat.

3.2. Objective 2: Assess the effects of feeding sunflower proteins treated with malic acid and heat on productive performance, diet digestibility, rumen and cecal fermentation, carcass characteristics and meat quality and fatty acid profile in growing lambs

In order to reach this objective two trials were conducted, one *in vitro* trial and one *in vivo* trial with growing lambs. Experiment 2 and 3 (Part I). "Effects of feeding rumen-protected sunflower seed and meal protein on feed intake, diet digestibility, ruminal and cecal fermentation and growth performance". Experiment 3 (Part II). "Carcass characteristics and meat composition and fatty acid profile in lambs fed sunflower protein protected against rumen degradation".

Experimental Diets: Two experimental concentrates with the same feed ingredients excepting SS and SM, which were included either untreated (control concentrate) or treated with malic acid and heat (MAH concentrate) were formulated. Ingredient and chemical composition of both concentrates is shown in Table 3. The MAH treatment consisted in spraying successively 15 kg of SS (previously ground through 4 mm screen) and 18.3 kg of SM in a concrete mixer with a 1 M malic acid solution (400 mL/kg of feed) using a sprayer. Both fractions were then mixed, spread on stainless steel trays, and allowed to rest for 15 min at room temperature. The mixture was dried in a forced air oven at 150 ºC for 2 h. Finally, the oven was turned off and the treated material was left in the oven to increase the thermal impact but avoiding oil exudation and burning. During the drying process, the feeds were manually stirred every 30 min during the 2 h of heating and every 60 min for the following 5 h. This process was repeated during three consecutive days to obtain the amount of SS and SM (45 and 55 kg, respectively) needed for preparing the MAH-concentrate. Untreated SS (ground through 4 mm screen) and SM were mixed homogeneously in this same

proportion to prepare the control concentrate. During the MAH treatment, samples of SS and SM were individually taken for being tested in the *in vitro* trial. The two concentrates were formulated with the same ingredients to contain (DM basis) 155 g/kg of crude protein.

Table 3. Ingredient and chemical composition of the experimental concentrates and barley straw

Item	Concentrate[1]		Barley
	CON	MAH	straw
Ingredients (g/kg fresh matter)			
Untreated Sunflower meal	109	-	
Treated Sunflower meal	-	109	
Untreated Sunflower seed	89.0	-	
Treated Sunflower seed	-	89.0	
Soybean mean	50.0	50.0	
Wheat	196	196	
Barley	264	264	
Corn	263	263	
CO_3Ca	22.4	22.4	
NaCl	4.8	4.8	
Mineral-vitamin premix	2.0	2.0	
Chemical Composition (% DM)			
Dry matter (DM; g/kg fresh matter)	899	895	923
Organic matter (g/kg DM)	941	940	891
Crude protein (g/kg DM)	156	153	29.0
Ethereal Extract (g/kg DM)	56.1	50.5	16.0
Neutral detergent fiber (g/kg DM)	184	191	719
Acid detergent fiber (g/kg DM)	67.1	72.6	380
Lignin (g/kg DM)	10.5	11.2	92.1

[1]Both sunflower seeds and meal were treated with malic acid and heat for protein protection in the MAH treatment.

3.2.1. *In Vitro* Trial:

Four adult rumen-fistulated sheep (67.8 kg ± 2.39 kg body weight) were used as rumen fluid donors for the *in vitro* incubations. Animals were individually housed in floor-pens and had free access to water and a mineral-vitamin block over the trial. Sheep were fed a mixed diet of grass hay and a commercial concentrate in 1:1 ratio at a daily rate of 45 g per kg of body weight$^{0.75}$ administered in two equal meals. The diet

contained 913, 150, 365, and 160 g of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), and acid detergent fibre (ADF) per kg, respectively. Samples of SS and SM either untreated or treated and from both concentrates were ground through a 1 mm screen before analysis of chemical composition and *in vitro* fermentation with buffered rumen fluid. Two *in vitro* trials were conducted on different days using the same methodology and in each of them there were four replicates per feed sample by using the ruminal fluid from each sheep as inoculum (1 vial per feed and inoculum). The first *in vitro* trial was performed to assess the gas production kinetics of the samples, whereas the objective of the second trial was to determine the main fermentation parameters and CH_4 production. Ruminal contents of each sheep were obtained before the morning feeding, strained through four layers of cheesecloth into thermal flasks, and immediately transported to the laboratory. The fluid of each sheep was independently mixed with a pre-warmed (39 ºC) culture medium (Goering and Van Soest, 1970) in a proportion 1:4 under CO_2 flushing. The medium of Goering and Van Soest (1970) was modified to avoid N supply by replacing the $(NH_4)HCO_3$ with $NaHCO_3$ and excluding the trypticase. Samples (200 mg of DM) of each feed were accurately weighed into 60 ml vials, which were filled up with 20 ml of the buffered rumen fluid using a peristaltic pump (Watson-Marlow 520UIP31; Watson-Marlow Fluid Technology Group, Cornwall, UK), sealed with rubber stoppers, and incubated at 39 ºC for 120 h. Gas production was measured at 2, 4, 6, 9, 12, 16, 21, 25, 30, 35, 48, 60, 72, 96, and 120 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe. The gas produced at each measurement time was released to prevent gas accumulation. Additional vials without substrate (blanks; two per inoculum) were included to correct the gas production values for the gas released from endogenous substrates. The second *in vitro* trial lasted for 12

h. Gas production was measured and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH_4 concentration. Vials were uncapped, their content was homogenized, the pH was immediately measured (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain), and 3 ml of vials content were mixed with 3 ml of 0.5 M HCl for analyses of volatile fatty acid (VFA) and NH_3-N concentrations. Samples were frozen at −20 ºC until analysis.

3.2.2. *In Vivo* Trial:

Twenty four male Lacaune lambs (14.2 ± 0.35 kg body weight) were distributed into two homogeneous groups according to their body weight; each group was randomly assigned to one of the two experimental concentrates (CON and MAH). Lambs were housed in individual pens (1 m × 1 m) with slatted floors, placed at 1 m above the floor, and equipped with two feeders and an automatic drinker. Pens were placed in a temperature-controlled room set at 20 ºC. During the experiment (40 days), lambs were fed *ad libitum* concentrate and barley straw, and had free access to fresh water. Concentrate and straw intake was monitored twice per week and all lambs were weighed weekly. Samples of offered concentrate and straw were taken weekly for analysis of chemical composition.

After 30 days of trial, diet digestibility was measured in 10 lambs per treatment. Trays (1 m × 1 m) provided with holes were placed under the slatted floor of each pen for feces collection. Total feces voided by each lamb in 24 h were quantitatively collected for six days. An aliquot (10%) of total fecal output was collected each day for digestibility determination. Daily samples of feces were pooled to form a composite sample for each lamb and dried to constant weight before analysis. On days 0, 20, and the slaughter day, blood samples were collected from each lamb via jugular venipuncture into tubes containing EDTA immediately before feeding. Samples were

centrifuged (6000× g for 10 min at 4 ºC), and the plasma was immediately frozen (−20 ºC) until determination of urea-N and amino acid-N. Due to a problem during the storage of the samples taken on day 20, they could not be analyzed for urea-N, and values for this sampling are not reported.

After 39 days, lambs were weighed and slaughtered at a commercial slaughterhouse located 20 km far from the experimental farm on two different days (six lambs of each treatment per day). Lambs had free access to feed until about 45 min before slaughtering, and slaughtering and dressing followed the standard commercial practice. After removing the full gastrointestinal tract samples from rumen and cecal contents were immediately taken. The ruminal content was homogenized, a sample of about 300 g was filtered through four layers of gauze, and the pH of the fluid was immediately measured (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain). Then, 2 ml of fluid were mixed with 2 ml of 0.5 N HCl. In addition, 2 g of cecal content were weighed and mixed with 2 ml of 0.5 N HCl. Samples were immediately frozen at −20 ºC until analysis of NH_3-N and VFA concentrations. Finally, rumen papillae characteristics were evaluated as described by Carrasco et al. (2012). Briefly, a 10 × 10 cm rumen wall sample was collected from the ventral area of the rumen after rumen content sampling. The ventral area of the rumen was identified using the oesophagus and the spleen as physical references. Samples were washed with saline solution and the excess of solution was removed from the surface with tissue paper. Samples were then displayed on a white surface under an intense and homogeneous light to evaluate their color by assigning to each of them a score from 1 (pale) to 5 (dark). The evaluation was performed by four trained persons, who were blind to treatment allocation, and the average score was used for statistical analysis. In addition, pictures of all samples were taken and downloaded into a computer. Six 1-cm^2 areas

were marked in each picture and the ruminal papillae within each area were counted. Finally, 10 papillae were randomly selected in each sample to measure their length and width using a Mitutoyo® calibrator (Mitutoyo Corporation, Aurora, IL, USA) with a minimum resolution of 0.01mm.

The carcasses were weighed immediately after slaughter and weighed again after 24 h of airing in a cold chamber (4 ºC). In addition, the pH of the *longissimus dorsi* and *semitendinosus* muscles were measured at 0 and 24 h after slaughter using a penetration electrode adapted to a portable pH meter (Hanna Instruments pH meter HI-9025; Hanna Instruments SL, Eibar, Spain). At each time, two measurements were made for each muscle. Meat color was measured on *longissimus dorsi* and *rectus abdominis* muscles of each lamb, as well as on the subcutaneous tissue of the tail root, using a Minolta Spectophotometer CM-2500c (Minolta, Osaka, Japan) with illuminant D65, visual angle of 10° and measurement aperture of 8 mm. Three measurements were made on each location and values were averaged before statistical analysis. Calibration was performed as described by De la Fuente et al. (2014) by using standard white tiles prior to color measurements. The color coordinates were expressed following the CIELAB system (Comission Internationale de L'Eclairage, 2004) as L^* (brightness), a^* (red-green index) and b^* (yellow-blue index). In addition, chroma (C*) and hue (h*) values were calculated as $C^* = (a^{*2} + b^{*2})^{0.5}$ and $h^* = \tan^{-1}(b^* / a^*)$, respectively. Water-holding capacity of meat was measured as pressure loss using the procedure of Grau and Hamm (1953), and it was expressed as the percentage of expelled juice after compression. A sample of the *longissimus dorsi* of each lamb was taken, frozen and freeze-dried for analysis of chemical composition and fatty acid (FA) profile.

Carcass conformation measurements were made as proposed by Cañeque et al. (2004). Briefly, carcass width (CWD) was the widest carcass measurement at the ribs,

thoracic depth (CTD) was measured as the maximum distance between the sternum and the back of the carcass at the sixth thoracic vertebra, buttock width (BUW) was the widest buttock measurement in a horizontal plane on the hanging carcass), hind limb length (HLL) was the length from perineum to distal edge of the tarsus), and internal carcass length (ICL) was measured as the length from cranial edge of the symphysis pelvis to the cranial edge of the first rib. As proposed by Velasco et al. (2000), carcass compactness was calculated as [cold carcass weight/ICW] and buttock/leg index was estimated as [BUW/HLL].

In addition, carcass fatness was subjectively evaluated using the 1-5 points scoring system proposed by Colomer-Rocher et al. (1988). The dorsal fat thickness was measured at 4 cm from the carcass midline and at 4 cm from the caudal edge of the last rib with a digital calibrator (De la Fuente et al., 2014). The carcasses were split down by the dorsal midline, and the kidney knob and channel fat (KKCF) was assessed by visual observation and scored using a 1 to 3 scale (Colomer-Rocher et al., (1988). The left hind leg was separated, dissected and the length and weight of the metacarpus were recorded.

3.3. Objective 3: Evaluate the efficacy of a combined malic acid-heat treatment applied to sunflower seeds and sunflower meal on ruminal fermentation, bacterial composition and *in situ* degradation of both feeds

In order to reach this objective one *in vivo* trial with rumen-canulated sheep was conducted. Experiment 4. “Influence of feeding sunflower seeds and meal protected against ruminal fermentation on ruminal fermentation, bacterial composition and *in situ* degradability in sheep”.

Four rumen-cannulated mature ewes (79.5 kg ± 2.98 kg of body weight) were fed two experimental diets in a cross-over design with two periods of 24 days each. The two diets contained oat hay and concentrate in 40:60 ratio and were fed at 40 g dry matter (DM)/kg body weight$^{0.75}$ into six equal meals per day using automatic dispensers. Two concentrates were formulated with the same feed ingredients, excepting that sunflower seeds (SS) and dehulled solvent-extracted sunflower meal (SM) were included either untreated (control concentrate) or treated with malic acid and heat for protecting the protein against rumen degradation (MAH concentrate). Sunflower seeds and SM were ground at 4 and 2 mm size, respectively, before applying the protective treatment. The MAH treatment has been described by Haro et al. (2019) and consisted in spraying each feed independently with a 1 M malic acid solution (400 ml per kg of feed) and homogenising the mixture in a concrete mixer. Both feeds were then mixed, rolled out on stainless steel trays, and left 15 min at room temperature. The mixture was then introduced in a forced air oven at 150 °C for 2 h, the heating was turned off and the mixture was left into the oven overnight. During the heating, the mixture was manually homogenized every 30 min during the first 2 h of heating and every 1 h during the rest of the treatment. The procedure was repeated on different days until treating a total of 45 kg of SS and 55 kg of SM (45:55 SS:SM ratio). In addition, 1 kg sample of the mixture of SS and SM were submitted to the treatment and used for the *in situ* incubations. The treatment was also applied individually to 1 kg of SS and SM, which were heated in individual trays and used to measure the *in situ* degradability of each feed. The SS, SM and the mixture of both feeds were previously ground at 2 mm size to equal the particle size of all *in situ* tested feeds. The treatment of the samples used for the *in situ* incubations was carried out simultaneously with that of the SS and SM used for sheep feeding.

The two experimental concentrates contained (fresh matter basis) 264, 263, 196, 109, 89.0, 50.0, 22.4, 4.8 and 2.0 g of corn, barley, wheat, SS, SM, soybean meal, CO_3Ca, NaCl and mineral-vitamin premix per kg, respectively, and were pelleted (4 mm diameter). Both concentrates were formulated to contain 155 g of crude protein (CP) and 56 g of ether extract (EE) per kg of DM. Chemical composition of untreated and treated SS and SM, both concentrates and oat hay is shown in Table 4.

Table 4. Chemical composition (g/100 g dry matter unless otherwise stated) of sunflower seed (SS) and sunflower meal (SM) either untreated (control) or treated with malic acid and heat (MAH), and of the concentrates and oat hay fed to experimental sheep

Item	SS		SM		Concentrate[1]		Oat hay
	Control	MAH	Control	MAH	Control	MAH	
Dry matter (g/100 g	96.0	95.4	92.1	91.5	89.9	89.5	91.2
Organic matter	97.0	97.0	93.0	93.1	94.1	94.0	93.6
Crude protein	16.4	16.2	43.9	39.3	15.6	15.3	4.79
Ether extract	47.2	42.7	1.75	1.80	5.61	5.05	1.58
NDF	34.8	33.5	36.7	31.1	18.4	19.1	57.9
ADF	18.0	18.9	21.8	18.5	6.71	7.26	31.1
Lignin (sa)	6.93	7.20	5.67	5.89	1.05	1.12	5.78

[1] Both concentrates contained (fresh matter basis) 264, 263, 196, 109, 89.0, 50.0, 22.4, 4.8 and 2.0 g of corn, barley, wheat, SS, SM, soybean meal, CO_3Ca, NaCl and mineral-vitamin premix per kg, and SS and SM were either untreated (control) or MAH treated (MAH concentrate).

Sheep were individually housed in floor-pens and had free access to water and a mineral-vitamin block over the trial. Each 24-day experimental period included successively 10 days of diet adaptation, 9 days for performing *in situ* incubations, one day for measuring ruminal parameters, and two days for rumen empting. From day 6 and until the end of each period, 200 ml per day of a $(^{15}NH_4)_2SO_4$ solution (98 atoms%) were continuously infused into the rumen of each sheep (50 mg of ^{15}N/d) to label the ruminal bacteria.

The *in situ* degradation of untreated SS and SM was measured in the sheep fed the control diet, whereas the degradation of treated SS, SM and the SS-SM mixture (SSM) was measured in the sheep fed the MAH diet. Each feed (SS, SM and SSM) was incubated for 2, 4, 8, 16, 24 and 48 h in the rumen into nylon bags (11 × 7 cm; inner dimensions) with a pore size of 46 µm containing 3 g (fresh matter basis) of feed. Incubations were repeated on two different days in each sheep. For the untreated SS and SM, one bag of each feed for all incubation times were simultaneously placed into the rumen just before the first morning feeding (09:00 h) in each incubation run. For the treated feeds (SS, SM and SSM), the three tested feeds were randomly associated for these incubations. After being collected from the rumen, bags were washed with tap water and stored at –20ºC. After finishing the incubations, bags were thawed and washed with cold water three times for 5 min in a mini-turbine washing machine. The same washing procedure was applied to four bags of each tested feed to obtain the non-washout fraction (0 h value). Finally, bags were frozen at –20ºC once again, freeze-dried and immediately weighed to calculate DM degradation at each incubation time. These data were fitted with time to the model of Ørskov and McDonald (1979), and DM effective degradability (ED) was estimated considering both the particle rumen outflow rate (k_p) and the rate of particle comminution and mixing (k_c) following the method described by Arroyo and González (2013), which is based on generating composite samples representative of the total rumen outflow of the undegraded feeds. In this procedure, the residues for 0, 2, 4, 8, 16, 24 and 48 h of incubation are considered representative of the composition of the undegraded feeds outflowing the rumen in the intervals 0-1, 1-3, 3-6, 6-12, 12-20, 20-36 and 36-72 h after feeding, respectively. These composited samples were generated by mixing the incubation residues obtained at the different times in the proportions calculated as the quotient between the flow in each

interval and the total ruminal flow of the tested feed. The resultant composite samples were analyzed for ash, N, EE (lipids), neutral detergent fibre (NDF) and acid detergent fibre (ADF) and for ^{15}N abundance and the effective degradability (ED) of organic matter (OM), CP, lipids, NDF, ADF was determined as described by González et al. (2009) from the concentration of each fraction in the composite sample (X) and the feed (Y) and the value of by-pass of DM obtained by the integration method: ED = 100 – (X × DM by-pass/Y). The correction for microbial contamination was performed based in the ^{15}N abundance in the composite sample (CS) and in the SAB as follows:

$$\text{Microbial N} = [(^{15}N_{cs} - {}^{15}N_{feed})/(^{15}N_{SAB} - {}^{15}N_{feed})]$$

$$\text{Microbial DM} = \text{Microbial N} \times (N_{cs}/N_{SAB})$$

$$\text{Microbial OM} = \text{Microbial DM} \times (OM_{SAB} / OM_{cs})$$

$$\text{Microbial Lipids} = \text{Microbial DM} \times (\text{Lipids}_{SAB} / \text{Lipids}_{cs})$$

The ^{15}N abundance in the tested feeds was determined in the zero time incubation residues because only the insoluble fraction is subjected to microbial contamination.

The composited samples were used to determine the *in vitro* CP intestinal digestibility following the procedure described by Gargallo et al. (2006) as detailed by Belverdy et al. (2019). Three hundred mg of each sample were weighed in duplicate into Ankom R510 bags. Bags were incubated for 1 h in a Daisy^{II} incubator (Ankom Technology, Fairport, NY, USA) containing 0.1 N HCl buffer (pH 1.9) with 1 g/l of pepsin (P-7000, Sigma, St. Louis, MO, USA). The bags were taken out, washed under running with tap water, and further incubated for 24 h in a 0.5 M KH_2PO_4 buffer (pH 7.75) containing 3 g/l of pancreatin (P-7545, Sigma, St. Louis, MO, USA) and 50 ppm of thymol. Both incubations were performed at 39°C. Finally, bags were washed with

cold water in a turbine washing machine (2 cycles of 5 min each), dried at 40°C for 72 h, and weighed. Incubation residues were pooled by sample and sheep and analyzed for N concentration to calculate the *in vitro* intestinal digestibility of CP.

On day 21, samples of rumen fluid (about 20 ml) were collected hourly from 09:00 to 13:00 h by using a syringe attached to a 25 cm tube provided with a nylon-filter (46 μm pore size) at its end. The tube was inserted into the rumen through the cannula 1 h before starting the samplings. The pH of the rumen fluid was immediately measured (Crison Basic 20 pH-meter; Crisson Instruments, Barcelona, Spain) and 5 ml were mixed with 5 ml of 0.5 M HCl. Samples were frozen at −20°C until analysis of volatile fatty acid (VFA) and NH_3-N concentrations.

Finally, on days 23 and 24 of each experimental period, the rumen of sheep was manually emptied at 09:00 h immediately before feeding. Rumen contents were homogenized and a representative sample of about 1 kg was taken for isolation of liquid-associated (LAB) and solid-associated bacteria (SAB) by differential centrifugation as described by Rodríguez et al. (2000). The rumen of one sheep from each treatment was emptied each day, and after sampling the digesta was returned to the rumen.

3.4. Chemical Analyses

All chemical analyses were performed in duplicate. Chemical composition of feeds, refusals, feces, bacterial isolates, and *in situ* feed residues was analyzed according the AOAC (2005) procedures for DM (ID 934.01), ash (ID 048.13), and ether extract (ID 945.16), with the exception that EE content in bacterial isolates pellets was analyzed as described by Folch et al. (1957). Concentrations of NDF, ADF, and lignin were determined following the procedures of Van Soest et al. (1991), and Robertson

and Van Soest (1981), respectively, using an ANKOM^{220} Fiber Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and α-amylase was used in the sequential analysis of NDF, ADF, and lignin, and ash-free values are reported. Nitrogen was measured by the Dumas combustion method employing a Leco FP258 N Analyzer (Leco Corporation, St. Joseph, MI, USA) and the amount of acid detergent insoluble N (ADIN) was determined by analyzing the N content in the residue obtained after the treatment of the sample with acid detergent solution.

Concentrations of NH_3-N and VFA *in vivo* and *in vitro* were determined by the phenol-hypochlorite method (Weatherburn, 1967) and by gas chromatography (García-Martínez et al., 2005), respectively. The analysis of CH_4 in Experiments 1 and 2 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).

In experiment 3, plasma concentrations of urea-N were determined by the glutamate dehydrogenase enzymatic-spectrophotometric method of Gutman and Bergmeyer (1974), and those of total amino acid-N by the 2,4-dinitrofluorobenzene (DNFB) method described by Goodwin (1968). For moisture analysis of meat, 5 g of the *longissimus dorsi* muscle were homogenized in a crucible with sea sand, 5 ml of ethanol were added and the sample was dried at 102ºC. Extraction of lipids from the freeze-dried *longissimus dorsi* samples was performed in triplicate by the procedure described by Hanson and Olley (1963). Fatty acid methyl esters (FAME) of total lipids were obtained according to the method described by Morrison and Smith (1964), and were analyzed by gas chromatography using a gas chromatograph (Agilent Technologies 6890, Santa Clara, CA, USA) equipped with a flame ionisation detector

and an Omegawax 320 capillary column (30 m x 0.32 mm internal diameter; 0.25 mm film thickness) with polyethylene glycol as the stationary phase (Supelco, Bellefonte, USA). Chromatographic conditions had been detailed described by Díaz et al. (2011). Individual FAME were identified by comparing their retention times with those from a known standard (Supelco, Bellefonte, PA, USA), and results were expressed as percentage of total FA identified.

The ^{15}N abundance in feeds and *in situ* feed residues in Experiment 4 was determined using an elementary analyzer (Flash 1112, Thermo, Bremen, Germany) coupled in continuous flow to an isotope ratio mass spectrometer (Delta V, Thermo, Bremen, Germany).

3.5. Calculations and statistical analysis

Data of gas production in the *in vitro* experiments 1 and 2, were fitted with time using the exponential model: $Gas = PGP\ (1 - e^{(-c\ (t - lag))})$, where PGP is the asymptotic gas production, *c* is the fractional rate of gas production, *lag* is the time before starting gas production, and *t* is the time of gas measurement. The average gas production rate (AGPR) was defined as the rate between the incubation start and the time at which half PGP is reached and it was calculated as $AGPR = PGP\ c / [2\ (\ln 2 + c\ lag)]$. The amount of OM fermented (OMF) was calculated from acetate, propionate, and butyrate production in each vial following the equation proposed by Demeyer (1991): OMF (mg) = 162 × [μmol acetate / 2) + (μmol propionate / 2) + μmol butyrate].

In experiment 4, data on DM degradation were fitted with time (t) to the model of Ørskov and McDonald (1979): $y = a + b\ (1 - e^{-kd\ t})$, in which a represents the soluble fraction, b is the insoluble degradable fraction and k_d represents the fractional degradation rate of b. The undegradable fraction (r) was estimated as 1 – (a + b). Dry

matter effective degradability was calculated by mathematical integration considering either only k_p ($DMED_p$) or both k_p and k_c ($DMED_{pc}$) according to the following equations:

$DMED_p = a + b\ (k_d/(k_d + k_p))$; (Ørskov and McDonald, 1979)

$DMED_{cp} = a + b * (k_d/(k_d + k_p)) * ((k_d + k_p + k_c)/(k_d + k_c))$; (ARC, 1984)

Effective degradability values for other feed fractions (OM, CP, NDF, ADF and lipids) were only calculated as above indicated considering k_p and k_c jointly. According to the results of previous studies by our group performed in sheep fed similar diets, a value of 0.04/h was assumed for k_p (Rodriguez et al., 2000; González et al., 2002, 2003, 2014; Pereira and González, 2004) and 0.65/h for k_c (Arroyo et al., 2013; González et al., 2014; Díaz-Royón et al., 2016).

In Experiment 2, gas production and fermentation parameters were analyzed independently for each feed (SS, SM, and concentrates) using the PROC MIXED of SAS (2017) as a mixed model, in which the treatment was the fixed effect and the inoculum was considered as a random effect. In Experiment 3, data on feed intake, diet digestibility and growth performance were analyzed as a one-way ANOVA using the GLM procedure of SAS (2017). Plasma metabolites were analyzed using the PROC MIXED of SAS (2017) as a mixed model with repeated measures, in which the effects of the MAH treatment, sampling time, and the MAH treatment × sampling time interaction were fixed and animal was a random effect.

In Experiment 4, data on composition of bacterial isolates were analyzed as a factorial ANOVA using the PROC MIXED of SAS (SAS, 2017), including the fixed effects of diet (control and MAH), bacterial isolate (SAB and LAB), diet x bacterial isolate interaction and experimental period, and the random effect of the sheep. Rumen

parameters were analyzed using the PROC MIXED of SAS as a mixed model with repeated measures, in which the effects of the diet, sampling time, diet x sampling time interaction and experimental period were fixed and sheep was a random effect. The effect of MAH treatment on *in situ* degradability and *in vitro* intestinal digestibility of the tested feeds was analyzed independently for each feed as a mixed model in which the effects of the MAH treatment and the experimental period were fixed and that of sheep was random.

In all experiments, significance was declared at $p < 0.05$, whereas $p < 0.10$ values were considered as a trend.

IV. RESULTS AND DISCUSSION

4.1. Objective 1: Analyse the influence of including rumen-protected proteins in high-cereal diets on *in vitro* ruminal fermentation and CH_4 production

Experiment 1. "Protecting protein against ruminal degradation could contribute to reduce methane production".

The combined malic acid heat treatment was selected because its efficacy for protecting protein from ruminal degradation was proved in previous *in situ* and *in vitro* studies (Arroyo et al., 2011, 2013; Díaz-Royón et al., 2016; Vanegas et al., 2017a,b). Malic acid, as other acids, causes denaturation of proteins, reduces its solubility and provides effective protection against degradation by ruminal microorganisms. The treatment caused only minor changes in chemical composition of both SS and SM (Table 2), and the slight decreases observed in CP, NDF, ADF and ADL content in TR samples compared with the untreated samples might be due to a dilution effect associated with adding malic acid in the treatment.

The experimental diets were formulated to contain a high proportion of cereal grains (62.2 and 45.2% for low-and high-protein diets respectively) because Vanegas et al. (2017a) observed that the contribution of protein fermentation to CH_4 production was greater with a rapidly fermentable substrate (starch) than with a slowly fermentable substrate (cellulose). Diets with two different CP levels were formulated to analyse the possible interaction between treatment and CP level. Finally, the study examined the differences between diets at two incubation times (8 and 24 h), as sunflower protein is rapidly degraded in the rumen (Arroyo et al., 2013) and the influence of treatment might become less marked as incubation time increased.

There were no effects ($p > 0.05$) of either treatment or CP level on final pH (values ranged from 7.02 to 7.08, and from 6.63 to 6.71 at 8 and 24 h of incubation respectively). As shown in Table 5, no treatment × CP level interactions were observed for any determined parameter, excepting a trend for CH_4/total VFA ratio at 8 h incubation ($p = 0.09$) and for total VFA at 24 h ($p = 0.088$). After 8 h incubation, the fermentation of diets including rumen-protected SS and SM resulted in lower NH_3-N concentration ($p = 0.01$) compared with CON diets, which is consistent with the hypothesis that treatment reduced sunflower protein degradation. The lower proportions of isovalerate and valerate observed in TR diets also suggest a reduction of protein degradation, as these VFA are only generated in the degradation of branched-chain amino acids (Wallace and Cotta, 1988).

Table 5. *In vitro* rumen fermentation of diets containing sunflower seed and sunflower meal, either untreated (CON) or treated against rumen degradation (TR), and two protein levels after 8 and 24 h of incubation

Incubation time and item	Experimental diets[1]					p =		
	13CON	13TR	17CON	17TR	SEM	TR	Protein level	TR x Protein level
8 h								
Gas (ml)	30.7	31.7	27.2	27.5	1.85	0.754	0.067	0.856
CH_4 (ml)	1.53	1.46	1.67	1.49	0.045	0.018	0.093	0.255
NH_3-N (mg/l)	78.0	62.4	85.5	69.7	3.01	0.007	0.090	0.976
Total volatile fatty acid (VFA; mmol)	0.421	0.421	0.443	0.427	0.0138	0.566	0.329	0.592
Individual VFA (mol/100 mol)								
Acetate	51.4	51.2	52.8	51.3	0.57	0.177	0.215	0.276
Propionate	31.9	33.0	31.0	33.2	0.34	0.001	0.295	0.138
Butyrate	12.7	12.4	12.3	12.1	0.27	0.366	0.208	0.673
Isobutyrate	0.25	0.11	0.17	0.06	0.106	0.277	0.569	0.909
Isovalerate	1.34	1.10	1.46	1.19	0.099	0.027	0.315	0.902
Valerate	2.34	2.19	2.29	2.14	0.072	0.072	0.515	0.987
Acetate/propionate (Ac/Pr; mol/mol)	1.64	1.58	1.73	1.57	0.033	0.009	0.206	0.164
CH_4/VFA (ml/mmol)	3.66	3.52	3.87	3.55	0.040	0.001	0.030	0.089
OMAF (mg)[2]	37.1	37.0	38.7	37.5	1.17	0.594	0.394	0.614

Table 5. *In vitro* rumen fermentation of diets containing sunflower seed and sunflower meal, either untreated (CON) or treated against rumen degradation (TR), and two protein levels after 8 and 24 h of incubation (continued)

Incubation time and item	Experimental diets[1]					p =		
	13CON	13TR	17CON	17TR	SEM	TR	Protein level	TR x Protein level
24 h								
Gas (ml)	56.3	55.5	57.7	58.3	3.29	0.980	0.546	0.837
CH_4 (ml)	5.68	5.53	5.05	5.13	0.125	0.795	0.003	0.387
NH_3-N (mg/l)	138	125	177	149	4.7	0.002	<0.001	0.168
Total volatile fatty acid (VFA; mmol)	1.16	1.13	1.02	1.06	0.017	0.952	<0.001	0.088
Individual VFA (mol/100mol)								
Acetate	54.0	54.3	53.5	54.3	0.64	0.405	0.701	0.657
Propionate	25.8	25.9	24.8	25.2	0.36	0.495	0.051	0.693
Butyrate	14.9	14.7	15.3	14.2	0.43	0.176	0.984	0.344
Isobutyrate	1.55	1.55	1.88	2.02	0.100	0.472	0.003	0.506
Isovalerate	1.85	1.72	2.28	2.07	0.068	0.034	<0.001	0.592
Valerate	1.94	1.90	2.26	2.14	0.069	0.261	0.003	0.593
Ac/Pr (mol/mol)	2.17	2.16	2.20	2.21	0.057	0.976	0.478	0.906
CH_4/VFA (ml/mmol)	4.92	4.92	4.96	4.88	0.071	0.536	0.998	0.518
OMAF (mg)[2]	103	99.9	89.8	92.1	1.43	0.807	<0.001	0.101

[1] 13CON and 17CON: Diets with two levels of protein (130 and 170 g/kg dry matter) and containing either untreated (CON) or treated (TR) sunflower seed and sunflower meal to protect protein against rumen degradation. Each culture contained 200 mg of diet dry matter. [2] Organic matter apparently fermented estimated as described by Demeyer (1991).

Fermentation of TR diets produced lower CH_4 production ($p = 0.02$) compared with CON diets, but total VFA was similar ($p = 0.57$). The lack of differences in total VFA production indicates that the treatment of SS and SM did not negatively affect their DM fermentation, and that the reduced CH_4 production observed in TR diets was not a consequence of lower diet fermentability. In fact, there were no differences ($p = 0.59$) between TR and CON diets in the estimated amount of OMAF. Previous *in vitro* studies with protein as the only substrate (Vanegas et al., 2017a,b) or replacing non-protein N with protein-N with different substrates (Carro et al., 1999; Vanegas et al., 2017a,b) showed that CH_4 generation in ruminal fermentation was partly due to protein degradation, presumably due to the hydrogen generation in the fermentation of carbon skeletons resulting from amino acid deamination. Values of NH_3-N concentrations and those of CH_4 production at both incubation times were positively correlated ($p < 0.001$) for both low-protein ($r = 0.797$; $n = 16$) and high-protein ($r = 0.892$; $n = 16$) diets. A positive relationship between NH_3-N and CH_4 may just reflect different amounts of organic matter fermented in the vials, but in this study there were no differences in the OMAF between CON and TR diets; therefore, it seems that the lower CH_4 production observed for TR diets was due to the reduction in protein degradation.

The ratio CH_4/VFA can be used as an indicator of the efficiency of ruminal fermentation, as CH_4 represents an energy loss and VFA are the main energy source for the host animal and are also precursors for the synthesis of fatty acids and glucose. In agreement with a previous study in which providing protein-N to *in vitro* cultures of ruminal microorganisms containing a rapidly fermented substrate enhanced CH_4/VFA ratio (Vanegas et al., 2017a,b) CON-diets showed greater CH_4/VFA ratios ($p = 0.001$) than TR diets at 8 h incubation, thus indicating a more efficient fermentation in TR diets. In addition, fermentation of TR diets resulted in increased propionate proportion

(p = 0.001) and reduced isovalerate proportion (p = 0.03). Different *in vitro* studies (Carro and Ranilla, 2003; Tejido et al., 2005) have reported that malate supplementation increased propionate production, which can contribute to a reduction in CH_4 production, as malate acts as an electron acceptor and is converted to propionate by the succinate pathway. In contrast to that observed at 8 h of incubation, no effects of treatment (p = 0.18 to 0.98) were detected at 24 h, with the exception of a reduction in both NH_3-N concentrations (p = 0.002) and molar proportion of isovalerate (p = 0.03). As before discussed, these differences would indicate lower protein degradation in TR compared with CON diets. The reduction in NH_3-N concentrations for TR diets, relative to those in CON-diets, was 19.2 and 12.6% at 8 and 24 h, respectively (values averaged across CP levels), which indicates that protein protection was reduced with time, probably as a consequence of the continuous proteolytic activity of ruminal microbes.

There were no differences between high-and low-protein diets at 8 h incubation, with the exception of greater CH_4/VFA ratios (p = 0.03; 3.71 vs. 3.59 ml/mmol) and a trend (p < 0.10) to lower gas production (27.4 vs. 31.2 ml) and to greater CH_4 production (1.58 vs. 1.50 ml) and NH_3-N concentrations (77.6 vs. 70.2 mg/l) for high-protein diets than for low-protein ones. This would indicate that the substitution of cereal grains (wheat and maize) by SM to reach 170 g CP/kg in high-protein diets resulted in lower fermentation efficiency at 8 h of incubation. After 24 h of incubation, both CH_4 and VFA production were lower (p = 0.003 and 0.001 respectively) for high compared with low-protein diets, resulting in a lower amount of OMAF for the high-protein diets (p < 0.001; 91 vs. 101 mg). Ruminal fermentation of CP generates less CH_4 than carbohydrates (Jentsch et al., 2007), which could help to explain the lower CH_4 production observed for high-protein diets at 24 h as these diets contained less carbohydrates (698 g/kg) and more CP than low-protein diets (745 g of total

carbohydrates/kg; Table 2). The trend to greater CH_4 production observed at 8 h for high-protein compared with low-protein diets is difficult to explain, but could be related to greater CP degradation in these diets resulting in greater fermentation of carbon skeletons generated from amino acid deamination. This hypothesis is supported by the trend to increased NH_3-N concentrations in high-protein diets.

4.2. Objective 2: Assess the effects of feeding sunflower proteins treated with malic acid and heat on productive performance, diet digestibility, rumen and cecal fermentation, carcass characteristics and meat quality and fatty acid profile in growing lambs

Experiment 2 and 3 (Part I). "Effects of feeding rumen-protected sunflower seed and meal protein on feed intake, diet digestibility, ruminal and cecal fermentation and growth performance".

There were only slight differences in chemical composition between both concentrates. Compared with CON, the MAH concentrate contained 10% less ether extract (Table 3) and more NDF (3.8%) and ADF (8.2%). The lower ether extract content was mainly due to the reduction in the ether extract content of SS produced by the MAH treatment (from 475 to 427 g/kg DM), which was attributed partly to a dilution effect associated with adding malic acid in the protective treatment and partly to fat losses during the heating process, as partial oil exudation was observed during this process. The slight increases in NDF and ADF may be associated with the heat treatment of sunflower seeds, as previously reported for different feeds (Pereira et al., 1998; Mustafa et al., 2003).

4.2.1. *In Vitro* Trial

The effects of the MAH treatment on the *in vitro* fermentation of SS, SM, and both concentrates are shown in Table 6. Final pH ranged 6.28 to 6.77 and was not affected by MAH treatment for any substrate. Compared with the untreated SS, the MAH treatment increased PGP ($p = 0.001$) and AGPR ($p = 0.043$), which would indicate a stimulation of rumen fermentation. The greater VFA production and amount of OMF ($p = 0.026$ and 0.021, respectively) observed for MAH-treated SS are consistent with this idea and might be partly due to the direct fermentation of the added malic acid as malic acid is rapidly fermented by rumen microorganisms. In fact, Russell and Van Soest (1984) reported that a greater concentration of malic acid (7.5 mM) than that used in the present study (4.5 mM) was fermented *in vitro* within the first 12 h of incubation. In contrast, for SM neither AGPR nor VFA production were affected by MAH treatment, although a trend ($p = 0.058$) to greater PGP was observed. The concentrate including MAH-treated SS and SM had greater AGPR ($p = 0.020$) and tended to have greater ($p = 0.091$) fractional rate of gas production than the CON concentrate, but VFA production was not affected. The Variable effects of MAH treatment observed for the three substrates might be related to other additional effects of the treatment rather than malic acid fermentation itself. The increased fermentation observed in MAH-SS might be due to changes in their particle structure, which possibly improved microbial colonization. Thus, the partial oil exudation observed during the heat treatment may have reduced the potential toxic effects of free fatty acids on cellulolytic bacteria and consequently might have improved the microbial colonization of SS particles.

Table 6. Gas production parameters and *in vitro* fermentation parameters of sunflower seed, sunflower meal and concentrates containing both sunflower products either untreated (control) or treated with malic acid and heat (MAH) for protecting protein against ruminal degradation[1]

Item	Sunflower seed				Sunflower meal				Concentrate			
	Control	MAH	SEM[2]	p =	Control	MAH	SEM[2]	p =	Control	MAH	SEM[2]	p =
Gas production parameters[3]												
PGP (ml/g dry matter)	63.3	82.1	1.09	0.001	147	164	3.94	0.058	270	266	3.93	0.466
c (% / h)	4.40	4.56	0.52	0.937	5.48	4.98	0.17	0.117	5.60	5.88	0.08	0.091
Lag (h)	0.90	0.28	0.27	0.197	1.11	0.48	0.25	0.171	3.20	2.98	0.09	0.167
AGPR (ml/g dry matter)	1.79	2.52	0.15	0.043	5.36	5.69	0.26	0.430	8.57	8.83	0.04	0.020
Ruminal parameters												
CH_4 (ml)	1.69	1.96	0.092	0.100	3.84	4.07	0.359	0.424	5.59	5.95	0.221	0.330
NH_3-N (mg/l)	211	207	2.8	0.125	322	279	4.99	0.009	161	146	3.71	0.065
Total Volatile fatty acids	37.2	41.7	0.77	0.026	55.2	56.0	2.51	0.822	63.6	67.4	1.96	0.265
Individual VFA (mol/100ml)												
Acetate	67.7	65.5	0.18	0.003	66.3	63.6	0.38	0.015	62.8	61.5	0.40	0.349
Propionate	20.3	23.0	0.32	0.010	22.0	25.7	0.38	0.006	23.2	24.4	0.26	0.044
Butyrate	8.17	7.54	0.22	0.141	7.63	7.12	0.11	0.044	11.9	11.5	0.52	0.616
Minor VFA	3.87	4.08	0.111	0.292	4.13	3.63	0.178	0.143	2.89	2.68	0.235	0.585
Acetate/propionate	3.37	2.86	0.071	0.015	3.03	2.49	0.060	0.006	2.70	2.55	0.022	0.023
OMF (g/kg)	217	241	39.2	0.021	331	337	13.7	0.805	397	419	6.9	0.200

[1] Substrates (200 mg dry matter) were incubated with sheep ruminal fluid for 120 h for measuring gas production kinetics and for 12 h for measuring fermentation parameters (n = 4). Control concentrate contained untreated SS and SM and MAH concentrate contained MAH-treated SS and SM. [2] Standard error of the mean. [3] PGP: potential gas production; *c:* fractional rate of gas production; *lag*: time before the onset of gas production; AGPR: average gas production rate until half of PGP is reached; OMF: organic matter fermented estimated from VFA production as described by Demeyer (1991); minor VFA: calculated as the sum of isobutyrate, isovalerate and valerate proportions.

Vanegas et al. (2017a) applied the same protective treatment, with the exception that heating was applied for 1 h, to a different sample of SS and reported no effects on PGP, AGPR, and VFA production, but CH_4 production and NH_3-N concentrations were decreased. The lack of effect on CH_4 production observed in our study might be due to the increased fermentation of SS due to MAH treatment, as CH_4/VFA ratio was similar for both untreated and treated SS (2.27 and 2.35 mL/mmol, respectively). Concentrations of NH_3-N were decreased by 5.5%, but differences did not reach the significance level; however, a protein protection could not be excluded, as this could have been compensated with the increased fermentation observed for MAH-treated SS. Differences in the results of Vanegas et al. (2017a) and in the present study may be associated with the increased heating in the present study, not only due to the greater time of heating but also to the greater humidity in the oven due to the larger quantity of SS treated (about 47 kg in the present study and 500 g in the study of Vanegas et al. (2017a).

The trend to greater PGP ($p = 0.058$) and the lack of effect on both VFA production and OMF observed when SM was MAH-treated are in good agreement with the results reported by Vanegas et al. (2017a) for the same feed. The protective effect of MAH treatment against protein degradation indicated by these authors was supported by the reduced ($p = 0.009$) NH_3-N concentrations observed in our study for the MAH-treated SM compared with the untreated SM. The inclusion of MAH-treated SS and SM in the concentrate resulted in greater ($p = 0.020$) AGPR and a trend to lower ($p = 0.065$) NH_3-N concentrations compared with CON concentrate. In addition, the lack of effects on VFA production and OMF indicates that MAH treatment had no detrimental effect on ruminal fermentation.

The MAH treatment affected VFA profile of the three substrates (SS, SM, and concentrate) and both an increase ($p \leq 0.044$) in molar proportions of propionate and a decrease ($p \leq 0.023$) in acetate/propionate ratio was observed for all of them. In addition, a reduction ($p \leq 0.015$) in acetate molar proportions was observed for MAH-treated SS and SM ($p = 0.003$ and 0.015, respectively). The effects of malic acid or malate salts on VFA profile have been reported to vary with the incubated substrate (Tejido et al., 2005; Gómez et al., 2005), but an increase in propionate production has been observed in most *in vitro* studies because malate is an intermediate metabolite of the succinate pathway of propionate production by ruminal microorganisms (Gómez et al., 2005). Similar changes in VFA profile were observed by Vanegas et al. (2017a) for both SS and SM.

4.2.2. *In Vivo* Trial

As shown in Table 6, there were no differences between groups in concentrate, barley straw, and total feed intake, showing that the MAH treatment, which included malic acid at 1.06% of concentrate, did not negatively affect concentrate palatability. This agrees with previously reported results in growing lambs (Carro et al., 2006; Mungói et al., 2012) and beef cattle (Montaño et al., 1999; Carrasco et al., 2012), indicating that no negative effects of malic acid or malate salts on feed intake should be expected when they are included in the diet at levels up to 2.6%. Although lambs were fed barley straw *ad libitum*, the intake of barley straw was very low, ranging from 2.1 to 5.2% of total DM intake. This low intake of straw is consistent with that observed in previous studies in lambs under similar feeding conditions (Manso et al., 1998; Carro et al., 2006).

The MAH treatment did not affect final body weight of lambs, average daily gain, feed conversion rate, and carcass traits (Table 7), which is in agreement with the

lack of differences observed by Díaz-Royón et al. (2016) when applying the MAH-treatment to SM and spring pea protein. The present study and that of Díaz-Royón et al. (2016) used similar feeding systems (high-cereal concentrates and cereal straw fed *ad libitum*), lambs had similar initial and final weights, and the amount of malic acid included in the MAH-treated concentrates was also comparable (1.1%) but differed in the MAH-treated protein feeds included in the concentrate.

Table 7. Initial and final body weight, feed intake, average daily gain, feed conversion rate, carcass traits and diet digestibility of growing lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate		SEM[1]	p =
	Control	MAH		
Initial body weight (kg)	14.2	14.2	0.31	0.975
Final body weight (kg)	26.5	27.1	0.46	0.357
Feed intake (g/d)				
Concentrate	873	915	21.6	0.184
Straw	31.3	32.9	2.15	0.613
Total	904	948	2.1	0.178
Average daily gain (g/d)	314	329	14.8	0.473
Feed conversion rate (g/g)	2.98	2.92	0.161	0.766
Carcass traits				
Hot carcass weight (kg)	12.7	13.7	0.21	0.212
Cold carcass weight (kg)	12.4	12.8	0.21	0.198
Cold carcass yield (%)	46.8	47.2	0.88	0.736
Diet digestibility (g/kg)				
Organic matter	807	815	7.0	0.074
Crude protein	719	731	9.9	0.394
Neutral detergent fiber	506	505	19.9	0.962
Acid detergent fiber	393	383	21.7	0.335

[1] Standard error of the mean.

Organic matter digestibility tended to be greater (p = 0.074) in MAH-fed lambs than in the control group, but there were no differences in the digestibility of CP, NDF, and ADF. Most studies have shown a lack of effect of malic acid or malate salts

supplementation, as feed additives on diet digestibility in lambs Carro et al. (2006) and beef cattle Montaño et al. (1999), although Flores et al. (2003) observed an increase in diet digestibility by supplementing Manchega and Lacaune fattening lambs with disodium–calcium malate at 2 and 4 g/kg DM and Mungói et al. (2012) observed a reduction of diet digestibility by using the same product at 1 g/kg DM in the diet of Manchega fattening lambs. These discrepancies in the results may be related to differences in the composition of the concentrate used in the different trials. In addition, it should be considered that in the present study malic acid was not used as feed additive and the heat treatment might have modified its effect on rumen fermentation.

Blood concentrations of urea-N are markedly influenced by the amount of NH_3-N absorbed from the rumen and therefore they reflect the balance between protein degradation and the use of NH_3-N for microbial protein synthesis. However, urea-N concentrations also reflect the amount of urea produced by the liver from amino acid catabolism (Calsamiglia et al., 2010). As shown in Table 8, plasma concentrations of urea-N and amino acid-N were not affected by MAH treatment. Urea-N concentrations were steady over the trial, but amino-N decreased ($p < 0.001$) with advancing time. Eryavuz et al. (2003) also observed that plasma concentrations of amino-N in growing lambs decreased with age, which was attributed to the fact that protein requirements decrease with age and therefore the synthesis of amino acids in the liver is reduced.

Table 8. Plasma concentrations of urea-N and amino acid-N in lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH) at the start (0), middle (day 20) and end of the trial

Item	Treatment (TR)	Sampling day			SEM_{TR}[1]	SEM_{T}[1]	p =		
		0	20	Slaughter time			TR	Time	TR x Time
Urea-N [mg/100 ml]	Control	28.5	-	28.3	1.13	1.13	0.755	0.677	0.795
	MAH	28.4	-	27.3					
Amino acid-N [mg/100 ml]	Control	0.74b	0.66b	0.33a	0.060	0.073	0.500	<0.001	0.383
	MAH	0.87b	0.60a	0.34a					

[a-b] Within each parameter and row, different superscripts indicate differences among sampling times ($p < 0.05$; LSD test). [1] SEM_{TR}: standard error of the mean for treatment effect; SEM_{Time}: standard error of the mean for time effect.

The lack of effects of MAH-treatment on post-mortem VFA concentrations and VFA profile in the rumen and the cecum (Table 9) is in agreement with the results of Carro et al. (2006) who observed that supplementation of the concentrate fed to growing lambs with 0.4 or 0.8% of malate salts did not affect ruminal pH, total VFA concentrations or VFA profile measured after slaughtering. Similar results have been reported in beef cattle (Carrasco et al., 2012; Montaño et al., 1999) and dairy cows (Khampa et al., 2006) by supplementing malic acid or malate salts up to 2.64% of total diet. As discussed by Carro et al. (2006), it is possible that greater levels of malate would be necessary to detect significant effects on *in vivo* VFA production and diet degradability. Moreover, it has to be considered that, when determining VFA concentrations in the rumen, VFA proportions may be unrepresentative of net production (Udén, 2011).

Ruminal papillae characteristics were in the range reported by others for growing lambs fed high-cereal concentrates (Lane et al., 2000; Blanco et al., 2015) and were not affected by MAH treatment (Table 9). However, the colour of the ruminal epithelium was darker ($p = 0.003$) in the lambs fed the MAH-concentrate compared with the control group, which might be due either to a corrosive action of malic acid or to a greater abrasion of the MAH-treated sunflower husks. However, the lack of differences between groups in any other variable tested would indicate that ruminal absorption was not negatively affected by the MAH-treatment.

In summary, the MAH treatment was effective in increasing the *in vitro* fermentation of SS, reducing the *in vitro* protein degradability of SM, and modifying the VFA profile towards greater propionate production. However, under the conditions of the present study the inclusion of MAH-treated SS and SM in a concentrate for growing lambs did not influenced feed intake growth, diet digestibility or growth performance.

Table 9. Post-mortem fermentative parameters in the rumen and cecum of lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate		SEM[1]	p =
	Control	MAH		
Rumen				
pH	5.17	5.26	0.060	0.510
Total VFA (mM)	152	156	1.8	0.778
Individual VFA (mol /100 mol)				
Acetate	49.0	48.5	0.59	0.732
Propionate	41.0	41.6	0.61	0.757
Butyrate	5.87	5.98	0.48	0.919
Isobutyrate	0.48	0.48	0.139	0.931
Isovalerate	0.52	0.49	0.143	0.831
Valerate	2.52	2.49	0.251	0.915
Caproate	0.56	0.53	0.172	0.878
Acetate/propionate (mol/mol)	1.21	1.19	0.141	0.852
NH_3-N (mg/l)	51.8	52.1	2.11	0.989
Rumen wall characteristics				
Color[2]	1.71	2.78	0.267	0.003
Papillae length (mm)	3.94	4.01	0.157	0.779
Papillae numbers (per cm^2)	92.2	94.9	4.03	0.639
Cecum				
Total VFA (mM)	172	171	1.6	0.910
Individual VFA (mol /100 mol)				
Acetate	66.4	64.1	0.69	0.324
Propionate	19.4	20.1	0.61	0.647
Butyrate	11.5	13.2	0.50	0.172
Isobutyrate	0.36	0.37	0.117	0.922
Isovalerate	0.30	0.29	0.109	0.877
Valerate	1.40	1.34	0.203	0.776
Caproate	0.69	0.67	0.203	0.919
Acetate/propionate (mol/mol)	3.61	3.29	0.262	0.360
NH_3-N (mg/l)	76.6	63.1	1.97	0.485

[1] Standard error of the mean. [2] Color was scored from 1 (pale) to 5 (dark).

Experiment 3 (Part II). “Carcass characteristics and meat composition and fatty acid profile in lambs fed sunflower protein protected against rumen degradation”

As shown in Table 10, there were no effects ($p \geq 0.134$) of MAH concentrate on cooling losses, carcass conformation measurements and metacarpus weight and length, which is in agreement with the lack of differences between groups observed in the carcass weight. The measurements of carcass conformation and metacarpus were similar to those reported in other studies with lambs slaughtered at similar BW (Sañudo et al., 1997; Alcalde et al., 1999; Peña et al., 2005). The lack of differences in carcass conformation measurements is consistent with the similar composition of the diet and feed intake in both groups of lambs (Tables 3 and 7), as the energy and protein intake are main factors affecting lambs growth (Beauchemin et al., 1995; Peña et al., 2005). In agreement with our results, Díaz-Royón et al. (2016) applied the MAH treatment to SM and spring pea protein and observed no effects on the kidney-pelvic fat and dorsal fat.

However, in our study the MAH-fed lambs had greater ($p = 0.016$) dorsal fat thickness compared with the control lambs, which might indicate greater energy retention. In both, our study and that of Díaz-Royón et al. (2016), the same MAH treatment was used, similar diets (high-cereal concentrates and cereal straw) were fed, and lambs had similar initial and final weights. Therefore, the different response observed in the two studies in the dorsal fat might be related to the greater precocity of the Lacaune lambs (Vergara et al., 1999; Cañeque et al., 2004) used in our study compared with the “Entrefino” lambs used by Díaz-Royón et al. (2016).

Table 10. Cooling losses, carcass conformation measurements, and metacarpus weight and length of growing lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate		SEM[1]	p =
	Control	MAH		
Cooling losses (%)	2.63	2.43	0.172	0.590
Carcass conformation (cm)[2]				
CWD	19.3	19.3	0.34	0.973
CTD	20.9	22.0	0.48	0.134
BUW	53.8	54.1	0.54	0.634
HLL	22.3	22.1	0.35	0.693
RP	16.2	16.1	0.31	0.764
ICL	54.3	55.2	0.61	0.319
Carcass compactness	0.23	0.23	0.003	0.508
Buttock/leg index	2.42	2.46	0.056	0.550
Dorsal fat thickness (cm)	1.82	2.43	0.164	0.016
Carcass fatness[3]	2.22	2.25	0.160	0.906
Pelvic-kidney fat[3]	1.83	2.12	0.159	0.200
Metacarpus weight (g)	42.4	43.6	2.390	0.791
Metacarpus length (cm)	12.0	12.2	0.139	0.407

[1] SEM: standard error of the mean [2] CWD: carcass width; CTD: thoracic depth; BUW: buttock width; HLL: hind limb length; RP: rump perimeter; ICL: internal carcass length; carcass compactness was calculated as (cold carcass weight/ICW) and buttock/leg index was estimated as (BUW/HLL). [3] Measured according a 1 to 5 points scale (1: minimum score; 5: maximum score).

The pH values measured at 0 and 24 h were within the normal ranges for lamb meat (Cornforth, 1999), and were similar to those reported for light lambs of different breeds in other studies (Cañeque et al., 2004; Juárez et al., 2009). The color parameters of *longissimus dorsi* muscle and the subcutaneous tissue of the tail root were similar ($p \geq 0.107$) for both groups, but MAH-fed lambs had greater redness (a^*) and chromacity (C^*; $p = 0.010$ and 0.016, respectively) and tended ($p = 0.061$) to have greater yellowness (b^*) than the control lambs (Table 11). Meat color is affected by numerous factors such as breed, feeding, age and slaughter weight of the animal, exercise, slaughter stress, and meat storage conditions, among others (Sañudo et al, 1998; Priolo

et al., 2001). The color of lamb meat is crucial to ensure customer appeal and contributes strongly to the value of the product (Calnan et al., 2014), as consumers associate a bright meat color with freshness and quality (Wood et al, 2008). Therefore, the greater yellowness (*b**) and chromaticity (C*) of the MAH-treated meat might be favorable, as when the oxidation of the unsaturated fats takes place the yellow changes to a brown color and consumers associate that with the lack of freshness.

Table 11. pH and color values of different tissues of growing lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate		SEM[1]	p =
	Control	MAH		
pH				
Longissimus dorsi				
0 h	6.76	6.77	0.043	0.945
24 h	5.68	5.64	0.033	0.365
Semitendinosus				
0 h	6.59	6.44	0.067	0.130
24 h	5.77	5.75	0.044	0.743
Color				
Longissimus dorsi				
Lightness (L*)	38.5	36.6	1.48	0.385
Redness (a*)	8.60	10.2	0.77	0.147
Yellowness (b*)	12.4	13.3	0.43	0.144
Chromaticity (C*)	15.2	16.9	0.75	0.123
Hue* (H*)	55.8	53.1	1.65	0.264
*Subcutaneous tissue (*tail root)				
Lightness (L*)	64.4	63.0	1.66	0.554
Redness (a*)	1.80	3.36	0.656	0.107
Yellowness (b*)	11.9	12.0	0.99	0.933
Chromaticity (C*)	12.0	12.8	0.97	0.590
Hue* (H*)	67.5	60.1	13.6	0.703
Rectus abdominis				
Lightness (L*)	44.1	42.7	1.27	0.448
Redness (a*)	7.39	1.35	0.739	0.010
Yellowness (b*)	5.66	8.26	0.930	0.061
Chromaticity (C*)	9.62	13.4	1.01	0.016
Hue* (H*)	35.5	37.3	3.65	0.724

[1] SEM: standard error of the mean.

Neither the water-holding capacity of the meat nor its chemical composition were affected ($p \geq 0.327$) by MAH treatment (Table 12). Chemical composition values were in the range of those reported previously for light lambs of different breeds (Sañudo et al, 2000; De Almeida et al., 2015; Budimir et al., 2018).

Table 12. Water holding capacity and chemical composition of the *longissimus dorsi* muscle of growing lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate		SEM[1]	p =
	Control	MAH		
Water holding capacity (%)	66.8	67.8	1.00	0.506
Chemical composition (%)[2]				
Moisture	75.5	74.9	0.49	0.474
Protein	20.1	20.6	0.30	0.327
Fat	2.80	3.09	0.508	0.690
Ash	1.06	1.09	0.026	0.516

[1] SEM: standard error of the mean.

Fatty acid profile of the *longissimus dorsi* muscle is shown in Table 13. There were no differences ($p \geq 0.117$) between groups, with the exception of a trend ($p = 0.055$) to a greater proportion of C140:0 in the MAH-fed lambs compared with the control ones. The diet is one of the main factors affecting the meat FA profile (Santos-Silva et al., 2002; Aurousseau et al., 2004), which has important implications for human nutrition (Enser et al., 1998). The meat FA profile in our study was similar to that reported by others for lambs fed diets containing SS (Santos-Silva et al., 2003; De Almeida et al., 2015), being the C16:0 and C18:0 the most abundant saturated FA (SFA).

The addition of SS to the diet has been reported to increase the proportion of C18:1, C18:2, PUFA and n-6 FA in the meat of lambs (Santos-Silva et al., 2003; De Almeida et al., 2015). In our study, both groups were fed SS, but MAH-treated SS had a greater fat by-pass fraction than the untreated SS (results observed in the last experiment of this Thesis) and therefore a different FA profile of meat might be expected. Sunflower feeds (SS and SM) contain high amounts of PUFA, especially of C18:1 and C18:2 (Santos-Silva et al., 2003; De Almeida et al., 2015).

Table 13. Fatty acid profile of the *longissimus dorsi* muscle of growing lambs fed barley straw and concentrate containing sunflower meal and seed either untreated (control) or treated with malic acid and heat (MAH)

Item	Concentrate			
	Control	MAH	SEM[1]	p =
Fatty acid (% of total fatty acids)				
C10:0	0.10	0.10	0.009	0.781
C12:0	0.26	0.35	0.037	0.117
C14:0	3.23	3.60	0.129	0.055
C15:0	0.51	0.51	0.021	0.940
C16:0	21.5	21.2	0.34	0.553
C17:0	1.50	1.43	0.056	0.380
C18:0	8.18	9.09	0.460	0.177
C20:0	0.07	0.08	0.004	0.455
Total SFA	35.3	36.3	0.52	0.181
C14:1 n-9	0.15	0.15	0.010	0.763
C16:1 n-9	2.02	1.96	0.081	0.623
C17:1 n-9	0.92	0.87	0.044	0.430
C18:1 n-9	42.2	41.2	0.84	0.431
Total MUFA	45.3	44.2	0.89	0.410
C18:2 n-6	14.3	14.3	0.68	0.940
C18:3 n-3	0.15	0.16	0.008	0.808
C20:3 n-6	0.17	0.19	0.013	0.211
C20:4 n-6	4.35	4.29	0.245	0.780
C20:5 n-3	0.06	0.06	0.007	0.780
C22:5 n-3	0.29	0.31	0.023	0.570
C22:6 n-3	0.11	0.13	0.020	0.406
Total PUFA	19.4	19.4	0.86	0.970
n-6	18.8	18.8	0.85	0.998
n-3	0.61	0.66	0.044	0.442
n-6/n-3	31.4	29.9	1.88	0.563

[1] SEM: standard error of the mean.

The contents of MUFA in the *longissimus dorsi* in our study (42.2 and 41.2 g/100 g total FA for control and MAH concentrate, respectively) were similar to those reported by De Almeida et al. (2015) for lambs fed SS (42.1 and 43.9 g/100 g total FA for diets supplemented or unsupplemented with vitamin E, respectively), but PUFA concentrations in our study (19.4 g/100 g total FA for both groups) were greater (6.73

and 8.99 g/100 g total FA), despite that the level of SS inclusion in the diet was similar in both studies (89.0 and 80.1 g/kg, respectively). The greater PUFA proportion in our study might indicate a lower biohydrogenation of PUFA, possibly due to the low ruminal pH. In fact, it has been reported that low ruminal pH values decreases the population of ruminal microorganisms responsible for the isomerization and biohydrogenation of PUFA (Fuentes et al., 2009). Although ruminal pH was not measured in the study of De Almeida et al. (2015), the NDF content of the diet fed to lambs in their study (303 g NDF/kg) was greater than in our study (184 and 191 g/kg for control and MAH concentrate, respectively), and therefore greater pH values are expected. A low biohydrogenation rate in the control group in our study may also help to explain the lack of differences in FA profile despite of the increased fat by-pass fraction of the MAH-treated SS. The high content in C18:2 observed in our study compared with others feeding lambs with diets including SS (about 5-6 g/100 g total FA; Santos-Silva et al., 2003; De Almeida et al., 2015) seems to support this hypothesis. As a consequence, the proportion of n-6 FA in our study was greater than that reported in these studies and the n6/n-3 ratio reached values above 30. Decreasing the n6/n3 ratio in the diet has been considered favorable for human health, although some limitations when considering this ratio have also been risen more recently (Marventano et al., 2015).

In summary, the inclusion of SS and SM treated with malic acid and heat for protecting the protein against ruminal degradation in the concentrate of fattening lambs had no effect on carcass characteristics and meat quality, excepting that increased the amount of dorsal fat and the yellowness (*b**) and chromaticity (C*) of the *rectus abdominis*. More studies are needed to investigate the feeding conditions under which this treatment might be useful to increase lamb growth and meat quality.

4.3. Objective 3: Evaluate the efficacy of a combined malic acid-heat treatment applied to sunflower seeds and sunflower meal on ruminal fermentation, bacterial composition and *in situ* degradation of both feeds

Experiment 4. "Influence of feeding sunflower seed and meal protected against ruminal fermentation on ruminal fermentation, bacterial composition and *in situ* degradability in sheep".

As shown in Table 4, the MAH treatment produced only minor changes in chemical composition of SS, with the exception of a decrease in EE content which was attributed to fat losses by partial oil exudation from SS during the heating process. The slight decreases in CP, NDF and ADF content of the MAH-treated SM compared with the untreated SM were attributed to a dilution effect associated with adding malic acid in the MAH treatment, as previously discussed by Vanegas et al. (2017a). There were only small differences in chemical composition between both concentrates, although the MAH concentrate contained 10.1% less EE than the control concentrate, which is in agreement with the results observed for MAH-treated SS. In both concentrates, SM supplied about 35% of total CP, whereas SS supplied 66.6 and 67.0% of total EE for control and MAH concentrates, respectively.

Table 14 shows the post-prandial evolution on ruminal parameters in sheep fed the two experimental diets. No diet x time interaction was observed for any of the parameters measured. Feeding the MAH diet did not affect either ruminal pH or concentrations of NH_3-N and total VFA, but decreased ($p \leq 0.009$) molar proportions of acetate and propionate and increased those of butyrate ($p < 0.001$). As a consequence of the changes in VFA profile, sheep fed the MAH diet tended ($p = 0.098$) to have greater acetate/propionate ratios than control-fed sheep. As butyrate is mainly associated with protozoa activity (Newbold et al., 2015), these results might indicate a greater

development of protozoa in sheep fed the MAH diet, which might be associated with a greater resistance to degradation of the treated SS and SM resulting in a more continuous supply of soluble nutrients that might increase protozoa attraction and provide a more favorable ecological niche to extend their rumen residence time. Protozoa contribute markedly to ruminal deaminative activity (Willians and Coleman, 1988; Van Soest, 1994), and the presence of greater protozoa populations in MAH-fed sheep would help to explain the lack of significant differences in NH_3-N concentrations between diets, although NH_3-N concentrations were 7.1% lower in MAH than in control diet (74.2 and 79.9 mg/l, respectively; values averaged across sampling times).

Haro et al. (2019) fermented *in vitro* samples of the two concentrates used in this study with ruminal fluid from sheep fed a diet composed of 50:50 grass hay and a commercial concentrate, and observed no differences in total VFA production, but the MAH concentrate produced greater propionate proportions and tended to reduce NH_3-N concentrations compared with the control concentrate. The differences between both studies can be related to the different fermentation systems (*in vitro* vs. *in vivo*), but also to differences in microbial populations, as the *in vitro* study was conducted with ruminal fluid from sheep that had not been fed MAH-treated feeds. In addition, it has to be taken into account that VFA concentrations measured in the rumen are the balance between VFA production and VFA absorption and outflow from the rumen and cannot be representative of the actual VFA production.

Table 14. Post-prandial evolution of ruminal parameters in sheep fed the experimental diets[1]

Item	Diet	Time (h after feeding)				SEM_{Diet}	SEM_{Time}	p =		
		1	2	3	4			Diet	Time	Diet xTime
pH	Control	6.05	5.96	5.56	5.62	0.096	0.068	0.475	<0.001	0.717
	MAH	6.02	5.97	5.74	5.64					
NH_3-N (mg/l)	Control	81.3	86.2	79.0	73.1	6.49	4.59	0.228	0.392	0.655
	MAH	66.8	78.8	79.6	71.6					
Total volatile fatty acids (mM)	Control	89.5	100	117	113	7.17	5.07	0.987	0.029	0.573
	MAH	97.4	101	113	109					

Table 14. Post-prandial evolution of ruminal parameters in sheep fed the experimental diets[1] (Continued)

Item	Diet	Time (h after feeding) 1	2	3	4	SEM_{Diet}	SEM_{Time}	p = Diet	Time	Diet xTime
Individual VFA (mol/100 mol)										
Acetate	Control	60.6	59.4	58.6	59.2	0.33	0.24	0.007	<0.001	0.171
	MAH	59.6	57.9	58.5	59.0					
Propionate	Control	24.3	26.0	26.8	26.3	1.06	0.75	0.009	0.461	0.809
	MAH	23.2	25.2	23.9	23.5					
Butyrate	Control	11.2	11.4	11.4	11.1	0.90	0.64	<0.001	0.972	0.993
	MAH	13.7	14.3	14.1	14.0					
Isobutyrate	Control	0.88	0.77	0.70	0.83	0.077	0.055	0.769	0.319	0.914
	MAH	0.88	0.78	0.78	0.80					
Isovalerate	Control	1.26	1.07	0.98	0.99	0.095	0.067	0.848	0.142	0.724
	MaH	1.14	1.17	1.01	1.03					
Valerate	Control	1.33	1.34	1.47	1.49	0.051	0.036	0.345	0.056	0.621
	MAH	1.39	1.44	1.44	1.50					
Caproate	Control	0.35	0.25	0.26	0.27	0.028	0.020	0.560	<0.001	0.749
	MAH	0.40	0.26	0.26	0.27					
Ac/Pro (mol/mol)	Control	2.57	2.38	2.26	2.33	0.103	0.073	0.098	0.181	0.758
	MAH	2.60	2.44	2.46	2.54					

[1] Both diets had 40:60 oat hay:concentrate ratio, and concentrates contained sunflower seeds and sunflower meal either untreated (Control diet) or treated with malic acid and heat for protein protection (MAH diet).

As expected, ruminal pH dropped after feeding and its evolution was opposite to that of total VFA concentrations. In contrast, NH_3-N concentrations and VFA profile were not affected ($p \geq 0.142$) by sampling time, with the exception of slight reductions ($p < 0.001$) in molar proportions of acetate and caproate after feeding.

Chemical composition of ruminal bacteria was significantly affected by the diet (Table 15), and both the OM and lipid contents were lower in sheep fed the MAH diet than in those fed the control diet. The greater concentrations of total lipids in SAB compared to LAB agree well with previous results (Merry and McAllan, 1983; Legay-Carmier and Bauchart, 1989; Rodríguez et al., 2000; González et al., 2012), and had been attributed to a greater proportion of Gram-negative bacteria in SAB than in LAB (Rodríguez et al., 2000), as bacterial lipids are mainly phospholipids located in the bacterial cell wall and their concentration is markedly greater in Gram-negative than in Gram-positive bacteria (Cummins, 1989). Legay-Carmier and Bauchart (1989) observed that the inclusion of oil in the diet increased the lipid content of both SAB and LAB due to oil accumulation in bacterial vacuoles. In our study, lipid content was greater ($p < 0.001$) for both bacterial isolates in control-fed sheep compared with sheep fed the MAH diet. As indicated by others (Rodríguez et al., 2000; González et al., 2012), OM and lipid content are closely associated, so that lipid content expressed on OM basis was not affected by either diet or bacterial isolate ($p = 0.542$ and 0.371, respectively).

Feeding the MAH diet resulted in greater N ($p \leq 0.037$) content in ruminal bacteria, expressed either on DM or OM basis, and greater ($p = 0.005$) ^{15}N enrichment compared with the control diet. The greater ^{15}N enrichment in bacteria from MAH-fed sheep could be due to greater N capture from the ruminal NH_3-N pool and lower capture of the N resulting from feed degradation, which is consistent with a lower protein degradation of the MAH concentrate.

Table 15. Influence of the diet and bacterial fraction on chemical composition and ^{15}N enrichment of ruminal bacteria isolated from either solid (SAB) or liquid (LAB) rumen digesta[1]

Item	Bacterial isolate	Diet[2]		SEM	p =		
		Control	MAH		Diet	Bacterial isolate	Diet x Bacterial isolate
Dry matter (g/100 g)	LAB	91.3	91.1	0.28	0.346	<0.001	0.776
	SAB	94.4	94.0				
Organic matter (g/ 100 g dry matter)	LAB	80.1	76.6	0.98	0.011	0.121	0.797
	SAB	81.6	78.6				
Lipids (g/ 100 g dry matter)	LAB	17.9	12.4	0.65	<0.001	0.002	0.064
	SAB	19.5	16.8				
Lipids (g/ 100 g organic matter)	LAB	62.2	64.2	1.34	0.542	0.371	0.418
	SAB	62.1	61.8				
Nitrogen (g/ 100 g dry matter)	LAB	6.26	6.61	0.194	0.037	0.459	0.496
	SAB	5.97	6.60				
Nitrogen (g/ 100 g organic matter)	LAB	7.83	8.61	0.163	<0.001	0.059	0.408
	SAB	7.33	8.39				
^{15}N (% atoms excess)	LAB	0.295	0.326	0.0085	0.005	0.004	0.852
	SAB	0.259	0.294				

[1] Both diets had 40:60 oat hay: concentrate ratio, and concentrates contained sunflower seeds and sunflower meal either untreated (Control diet) or treated with malic acid and heat for protein protection (MAH diet).

The N content of was similar in both bacterial isolates when it was expressed on DM basis, but there was a trend ($p = 0.059$) to greater N content in LAB than in SAB when it was expressed on OM basis, which is in agreement with the larger machinery (ribonucleic acids, enzymes, ...) necessary to keep the greater duplication rate of LAB, which is consequence of the greater dilution rate of the liquid phase compared to passage rate of the solid digesta from the rumen (Bates et al., 1985, Rodríguez et al., 2000). The lower ($p = 0.004$) ^{15}N enrichment in SAB compared to LAB was probably due to the greater incorporation by SAB of N compounds (non-enriched NH_3-N, amino acids, amino sugars, …) derived from degradation of local feed particles, and agrees with most previous studies on this topic (González et al., 2012).

The MAH treatment of SS and SM modified the degradation kinetics of both feeds (Table 16), but no effects ($p \geq 0.373$) were detected on b and the undegraded fraction (calculated as a + b) for any feed. In contrast, MAH treatment decreased the fractional degradation rate of SS by 32.1% ($p = 0.004$) and that of SM by 49.3% for SM ($p = 0.055$). As protein denaturation is the basis of the MAH treatment, its efficacy should increase with augmented CP content in feed. Although the large oil content of SS might limit the access of the acid to the proteins, and therefore the effectiveness of the treatment, this effect was not clear in our study. The lack of previous thermal impact in SS compared with SM could possibly increase the effectiveness of the MAH treatment on SS. Previous studies using different protein feeds (Arroyo et al., 2013; Díaz-Royón et al., 2016) also showed a strong reduction in k_d caused by acid-heat treatments. In contrast to that observed by Arroyo et al. (2013) and Díaz-Royón et al. (2016), no changes in undegradable DM fraction were observed. The increase in the soluble fraction (a) produced by MAH treatment ($p = 0.062$ and 0.011 for SS and SM, respectively) might be due to the fermentation of the added malic acid, as previous

studies have reported a rapid fermentation malic acid (Russell and Van Soest, 1984; Vanegas et al., 2017b). Compared with the untreated feeds, the MAH treatment reduced the ED of DM of both SS and SM, either calculated using only k_p ($DMED_p$) or k_p and k_c ($DMED_{pc}$; Table 16). Compared with $DMED_{pc}$, the use of only k_p to calculate the effective degradability of DM underestimated the values by 4.2 and 4.1% for control and MAH samples for SS, respectively, and by 3.8 and 3.3% for SM, respectively.

Table 16. Effects of the protective treatment (MAH) on apparent rumen degradation kinetics and effective degradability of dry matter of sunflower seed and meal, calculated by mathematical integration considering either only the particle rumen outflow rate (k_p; $DMED_p$) or both k_p and the rate of particle comminution and mixing (k_c; $DMED_{pc}$)[1]

Feed and item[2]	Control	MAH	SEM	p =
Sunflower seed				
a (%)	11.7	13.4	0.31	0.062
b (%)	69.4	67.8	1.37	0.509
r (%)	19.0	18.8	1.06	0.932
K_d (%/h)	6.61	4.49	0.095	0.004
$DMED_p$ (%)	54.8	48.9	0.19	0.002
$DMED_{cp}$ (%)	57.2	51.0	0.19	0.002
Sunflower meal				
a (%)	15.0	20.0	0.37	0.011
b (%)	61.2	59.3	2.18	0.601
r (%)	23.8	20.7	1.93	0.373
K_d (%/h)	9.38	4.76	0.80	0.055
$DMED_p$ (%)	57.6	50.4	0.58	0.013
$DMED_{cp}$ (%)	59.9	52.1	0.58	0.011

[1] Control: untreated samples; MAH: samples treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 2 h. [2] *a:* soluble fraction; *b*: insoluble degradable fraction; *r*: undegradable fraction estimated as 1 – (a + *b*); k_d: fractional degradation rate of *b*.

Table 17, shows the values of microbial contamination of the composite residues of the *in situ* incubations, which was low for both SS and SM samples. In general, microbial contamination was lower for SS than for SM, with the exception of N contamination for MAH-treated SM. The microbial contamination of SM in our study

was intermediate between those reported by Arroyo et al. (2013) and by Díaz-Royón et al. (2016) for the same feed, who obtained 20.8 and 69.8 g/kg residual N, respectively, and 5.49 and 39.4 g/kg residual DM, respectively. The low microbial contamination observed in our study for SS and SM agrees well with the high lignification of the NDF of both feeds, as husks are a great proportion of the composite incubation residues. Highly-lignified tissues are a barrier to feed degradation by ruminal microorganisms and limit the development of microcolonies (Cheng et al., 1984; Chesson and Forsberg, 1988). The lower values of microbial contamination for SS compared with SM are consistent with the lower NDF content of SM (Table 4). In addition, the high oil content of SS could limit microbial colonization due to the toxic effect of free fatty acids on ruminal microbiota, especially of unsaturated fatty acids (Palmquist and Jenkins, 1980).

Table 17. Microbial contamination of ruminal undegraded composite samples of sunflower seed (SS) and meal (SM) samples incubated *in situ* in the rumen of sheep[1]

Item and feed	Control	MAH	SEM	p =
N (g/kg residual N)				
SS	30.2	25.0	2.45	0.276
SM	33.8	14.3	2.24	0.025
Dry matter (g/kg of residual dry matter)				
SS	8.23	6.15	0.253	0.028
SM	20.2	12.4	0.922	0.027
Organic matter (g/kg of residual organic matter)				
SS	6.83	4.93	0.257	0.035
SM	17.0	10.1	0.84	0.028
Crude fat (g/kg of residual crude fat)				
SS	4.35	2.65	0.182	0.022

[1] Composite samples were generated by mixing the incubation residues obtained at different incubation times in the rumen in the proportions calculated as the quotient between the flow in each incubation interval and the total ruminal flow of the tested feed (see text for description of calculations).

The MAH treatment was associated with a significant reduction of the microbial contamination (Table 17), although differences did not reach the statistical value (p =

0.276) for N in SS. Microbial contamination of SS was reduced by 17.3, 25.5, 28.1 and 38.9% for N, DM, OM and crude fat, respectively, whereas contamination of SM was reduced by 57.7, 38.5 and 40.9% for N, DM and OM, respectively, by applying the MAH treatment. As the k_d rate was reduced by the MAH treatment (Table 16), greater proportions of incubation residues from the short incubation times, which were subjected to less microbial colonization (González et al.,1998; Rodríguez and González, 2006), were included in the composite samples. The reduction in the k_d rate (higher in SM) with the MAH treatment implies the inclusion in the composite residue of higher proportions of residues corresponding to short incubation times subjected to less colonization (González et al.,1998; Rodríguez and González, 2006). The lack of correction for microbial contamination, joined with the use of only k_p for calculating the effective degradability, resulted in underestimation of the DM effective degradability by 5.12 and 4.44% for control and MAH-treated samples of SS, respectively, and by 4.80 and 4.58% for SM, respectively.

For both SS and SM, the MAH treatment reduced ($p \leq 0.023$) the effective degradability of all tested fractions with the exception of ADF (Table 18). As a result, the MAH treatment increased the by-pass CP fraction (values corrected for the microbial contamination) by 19.1 and 120% for SS and SM, respectively, and by-pass crude fat fraction of SS was augmented by 34%. As already discussed, the MAH treatment acts mainly on the protein fraction, which is in agreement with the greater increase of by-pass CP observed for SM compared with SS. The increase in the by-pass CP for SM was intermediate between those reported by Arroyo et al. (2013) and by Díaz-Royón et al. (2016), both using a sample of SM containing more degradable proteins than the one used in the present study. There were also some differences in the MAH treatments applied in these studies, as Arroyo et al. (2013) used the same dose of

malic acid and temperature (150 ºC) as in our study but heating lasted for 6 h, whereas Díaz-Royón et al. (2016) applied the same dose of malic acid but reduced to half the volume of solution and heating was set up to 120 ºC for 1 h. In all of these experiments, the treated feeds were allowed to stand in the stove overnight. The comparison of our results with previous ones seems to indicate that SM would benefit from a greater thermal impact than that applied in this study.

The lower efficacy of the MAH treatment on SS, compared with SM, could be due to the difficulty of diffusion of the malic acid solution into the SS particles due to their high oil content; however, the by-pass crude fat fraction was considerably increased (from 336 to 450 g/kg, values corrected for microbial contamination), which would be of especial value for high-production ruminant diets. Consequently, the MAH treatment of SS would reduce the negative effects of fat on ruminal fermentation, especially when high-forage diets are fed. Díaz-Royón et al. (2016) compared the effects of the MAH treatment on spring pea and SM and observed that the efficacy of the MAH treatment in increasing the supply of by-pass protein was reduced by decreasing the feed CP content, due to the greater proportions of other feed fractions (i.e. fiber) that are much less affected by the MAH treatment compared with CP. The reduction of DM, OM, CP and NDF degradation observed for both SS and SM by applying the MAH treatment (Table 14) is in agreement with the decrease in CH_4 production observed previously in the *in vitro* fermentation of both feeds (Vanegas et al., 2017a,b; Haro et al., 2018).The low values of NDF and ADF degradability of SS and SM are consistent with the high lignification of sunflower husks.

Table 18. Effects of the protective treatment (MAH) and of correcting for microbial contamination of the *in situ* incubation on estimates of effective degradability of different fractions of sunflower seed (SS) and meal (SM)[1]

Item and feed	Control		MAH		SEM	p =	
	Apparent	Corrected	Apparent	Corrected		MAH	Correction
Dry matter (g/kg)							
SS	572	57.5	510	513	1.6	< 0.001	0.088
SM	599	607	521	527	4.9	< 0.001	0.200
Organic matter (g/kg)							
SS	567	570	501	503	1.7	< 0.001	0.153
SM	581	588	496	501	4.8	< 0.001	0.252
Crude protein (g/kg)							
SS	734	742	686	693	6.72	< 0.001	0.281
SM	797	804	563	569	20.1	< 0.001	0.752
Neutral detergent fibre (g/kg)							
SS	382	-	252	-	14.1	0.023	-
SM	211	-	118	-	9.64	0.021	-
Acid detergent fibre (g/kg)							
SS	288	-	183	-	23.4	0.087	-
SM	140	-	90.3	-	14.31	0.131	-
Crude fat (g/kg)							
SS	663	664	549	550	6.9	< 0.001	0.852

[1] Control: untreated samples; MAH: samples treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 2 h.

The values of *in vitro* intestinal digestibility of by-pass CP for untreated and MAH-treated SS and SM are shown in Table 19. As microbial contamination of incubation residues was low, values were not corrected for the possible disappearance of rumen microorganisms attached to feed particles due to the enzymatic digestion. The *in vitro* intestinal digestibility of CP of the untreated SM (832 g/kg) was greater than the values (ranging from 699 to 801 g/kg) determined for samples of semi-decorticated SM using the mobile nylon bag technique in previous studies by our group (Gonzalez et al., 1999), Arroyo et al., 2013; Díaz-Royón et al., 2016), but greater values had been reported by NRC (2001) and INRA (2007) (900 and 890 g/kg, respectively). The *in vitro* intestinal digestibility of by-pass CP for the untreated SS (601 g/kg) was also lower than the 800 g/kg proposed by INRA (2007). The MAH treatment increased the *in vitro* intestinal digestibility of by-pass CP by 75.4 and 91.0% for SS and SM, which is in agreement with the results from previous studies applying malic acid-heat treatments to SM (Arroyo et al., 2013; Díaz-Royón et al., 2016). As indicated by Gonzalez et al. (1999), a reduction in ruminal degradability and degradation rate also produces a decrease in the amount undigestible compounds in the by-pass fraction, and therefore an increase in the intestinal digestibility may be expected. The MAH treatment also increased ($p = 0.041$) total CP digestibility of SS by 3.11%. The slightly reduction of total CP digestibility of MAH-treated SM (0.45%; $p < 0.001$) would indicate that the MAH treatment had no negative effect on CP availability.

Table 19. Effects of the protective treatment (MAH) on *in vitro* intestinal digestibility of by-pass protein (CP), intestinal digested fraction, and total digestibility of CP of sunflower seed (SS) and meal (SM)[1]

Item and feed	Control	MAH	SEM	p =
In vitro intestinal digestibility of by-pass CP (g/kg)				
SS	601	754	9.9	0.008
SM	832	910	1.1	< 0.001
Intestinal digested crude protein (g/kg)				
SS	160	236	05.4	0.010
SM	169	399	24.5	0.022
Total digestibility of CP (g/kg)				
SS	894	922	4.1	0.041
SM	966	962	0.02	< 0.001

[1] Control: untreated samples; MAH: samples treated with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 2 h.

As shown in Figure 4, MAH treatment was more effective on the SSM mixture than on SS and SM when both feeds were treated individually. In order to analyze the possible differences between the treatment of each feed individually and the SSM mixture, the results obtained for the MAH-treated SSM sample were compared with those obtained by applying to the SSM mixture the individual DM degradability values obtained for MAH-treated SS and SM samples. Compared with the calculated values, the measured DM degradation kinetics of SSM resulted in a significant increase of the soluble fraction (*a*; 18.3 *vs*. 17.0%; p = 0.013; SEM = 0.173), with no differences either in the insoluble but potentially degradable fraction (*b*; p = 0.203; 57.7 *vs.* 63.1%; p = 0.203; SEM = 2.37) or in the undegradable fraction (*r;* p = 0.286; 24.0 *vs.* 19.9%; SEM = 2.26). The most important difference was observed in the fractional degradation rate (*kd*), being the measured values lower than those calculated (p = 0.043; 3.76 vs. 4.65% / h; SEM = 0.187). As a result of these differences, there was a trend to lower values of the DM effective degradability (corrected for microbial contamination and calculated considering *kp* and *kc*) for the measured values compared with those calculated (p =

0.077; 48.0 *vs.* 52.1%; SEM = 1.09). Similar differences were observed for the effective degradability of the OM (p = 0.090; 46.1 *vs* 50.2%; SEM = 1.17), and the differences reached statistical significance for CP (p = 0.018; 53.6 *vs*. 59.8%; SEM = 0.93). The lower degradation observed for the SSM, as compared with the individual treatment of each feed, could not be attributed to a lower microbial colonization of the SSM mixture, as the contamination values measured in this sample (9.61 and 7.84% for DM and OM, respectively) are similar to those calculated as weighted mean for both MAH-treated feeds for DM (9.60%; p = 0.981; SEM = 0.74) and for OM (7.69%; p = 0.872; SEM = 0.59). Microbial contamination values for N showed large differences, but values did not differ (14.7 *vs*. 16.8%; p = 0.103; SEM = 0.63). Heating caused a partial exudation of the oil of SS that was partially adsorbed on SM particles and this "frying" effect might have caused a greater thermal impact, helping to explain the greater resistance to degradation observed in the SSM mixture. If this hypothesis were true, spraying oil on MAH-treated protein feeds after the application of the malic acid solution but before heating would be a means to increase the efficiency of the MAH treatment and to supply protected fat. However, the lack of negative effects of this method on rumen fermentation should be confirmed. Despite the greater by-pass CP content in the SSM sample (compared with the calculated values), there were no differences in the *in vitro* intestinal digestibility values (p = 0.713; 884 *vs*. 878 g/kg for the measured and calculated values, respectively; SEM = 11.6). As a consequence, the SSM sample had a greater content of intestinal digestible CP (p = 0.031; 416 *vs.* 360 g/kg; SEM = 10.4) but total digestibility of CP did not differ between measured and calculated values (p = 0.167; 945 *vs.* 951 g/kg; SEM = 2.4).

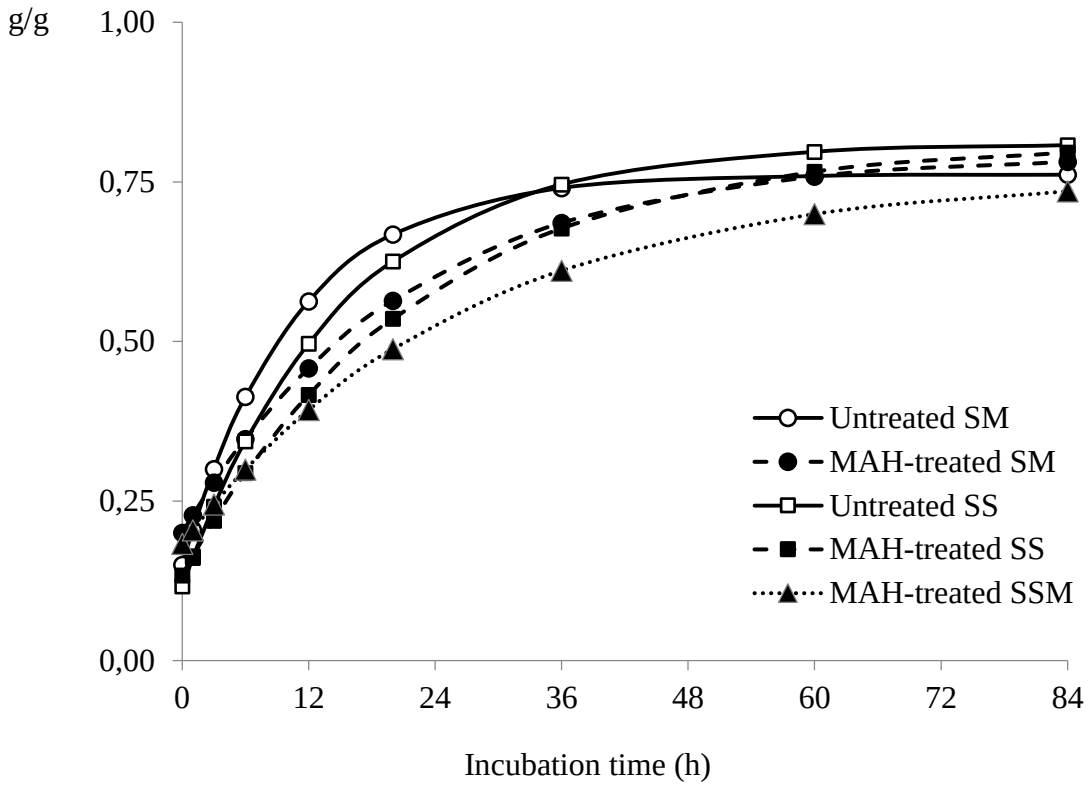

Figure 4. Dry matter degradation kinetics of sunflower meal (SM) and seed (SS) either untreated or treated (MAH) with a 1 M solution of malic acid (400 ml/kg feed) at 150°C for 2 h, and of a MAH-treated 45:55 SS:SM mixture (SSM)

In conclusion, the MAH treatment decreased the ruminal degradation of both tested feeds increasing the supply of by-pass protein by 19.1 and 120% for sunflower seeds and meal, respectively, and by 33.9% the by-pass fat for sunflower seeds. The simultaneous heating of a mixture of both feeds augmented the efficacy of the MAH treatment, increasing by 15.6% the content of intestinally digested protein compared with that obtained by applying the MAH treatment to sunflower seeds and meal individually. The results also confirm the previous observation that the effectiveness of MAH treatment decreases with reduced feed protein content. The inclusion in the diet of sheep of both treated feeds increased the nitrogen content of ruminal bacteria, and had not negative effect on ruminal fermentation.

V. CONCLUSIONS

1. The inclusion of sunflower seeds and sunflower meal treated with a combined malic acid-heat treatment in high-cereal diets resulted in lower *in vitro* CH_4 and NH_3-N production at early stages of fermentation (8 h) without decreasing the amount of organic matter apparently fermented. A greater fermentation efficiency of the diets including rumen-protected sunflower feeds was observed at 8 h of incubation, as indicated by the lower CH_4/volatile fatty acids and acetate/propionate ratios than for diets with unprotected protein.
2. The treatment of sunflower seeds and sunflower meal with malic acid and heat for protecting the protein against rumen degradation was effective in increasing the *in vitro* fermentation of sunflower seeds, reduced the *in vitro* protein degradability of sunflower meal and modified the volatile fatty acid profile towards greater propionate production, indicating the efficacy of this treatment.
3. The inclusion of sunflower seeds and sunflower meal treated with a combined malic acid-heat treatment in a concentrate for growing lambs did not influenced feed intake, growth performance or rumen and cecal post-mortem fermentation, although tended to increase the digestibility of the organic matter and increased the content of dorsal fat and the redness of the *rectus abdominis* muscle. However, no effects were observed on any carcass measurement and composition, and fatty acid profile of the l*ongissimus dorsi* muscle, and therefore the treatment cannot be recommended in the practice under the conditions of this study.
4. The treatment of sunflower seeds and sunflower meal with malic acid and heat decreased the *in situ* ruminal degradation of both feeds and increased the supply of by-pass protein by 19.1 and 120% for sunflower seeds and meal, respectively.

The heating of a mixture of both feeds seems to reinforce the protective effect and increased the intestinal digestible protein content by 15.6% compared with the individual treatment of both feeds. The practical circumstances under which this combined malic acid-heat treatment can improve animal performance should be further investigated.

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