GERMÁN DARÍO RAMÍREZ ZAMUDIO

EFEITO DA RESTRIÇÃO PROTEICA NA DIETA SOBRE A QUALIDADE DA CARNE DE OVELHAS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do programa de Pós-Graduação em Zootecnia, para obtenção de título de *Magister Scientiae.*

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APROVADA: 21 de fevereiro de 2017.

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(Coorientadora)

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BIOGRAFIA

Germán Darío Ramírez Zamudio, filho de Jinny Zamudio Peréa e José Didier Ramírez Cabezas, nasceu em 3 de março de 1986, na cidade de Villagarzón, Putumayo-Colômbia.

Ingressou no curso de graduação em Medicina Veterinária e Zootecnia na Universidade do Tolima, no ano de 2006, graduando-se como Médico Veterinário e Zootecnista em setembro de 2012.

Trabalhou como consultor técnico em criação de avestruz, no Fondo Ganadero del Tolima (Colômbia), durante os anos 2012 – 2013. Logo, foi consultor técnico em produção de gado de corte do Criadero Vista Hermosa (Colômbia), durante os anos 2012-2014.

Em agosto de 2015, ingressou no curso de Mestrado em Zootecnia, no Programa de Pós-Graduação em Zootecnia da Universidade Federal de Viçosa. Submeteu-se à defesa de dissertação em 21 de fevereiro de 2017.

SUMÁRIO

RESUMO

RAMÍREZ, Germán Darío Zamudio, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Efeito da restrição proteica na dieta sobre a qualidade da carne de ovelhas.** Orientador: Mário Luiz Chizzotti. Coorientadores: Cristina Mattos Veloso e Marcio de Souza Duarte.

Sabe-se que as calpaínas são as proteases mais importantes que atuam na regulação de síntese e degradação do musculo *in vivo*, como na transformação do musculo em carne, fato que pode ser afetado pela restrição de nutrientes. Por conseguinte, o objetivo deste estudo foi avaliar o efeito da redução abrupta de proteína na dieta sobre as caraterísticas organolépticas da carne de ovelhas durante a fase final de acabamento em confinamento. Foram utilizadas 14 ovelhas cruzadas (Dorper x Santa Inês), com peso corporal médio de 39,3 $\pm$ 9,4 kg, que foram confinadas na fase de acabamento por 46 dias. Ao início do experimento, todos os animais foram divididos em dois grupos, de forma randomizada, e receberam a mesma dieta contendo 13,3% de proteína bruta durante 32 dias. No 33º dia experimental um grupo (n=7) foi submetido a uma dieta com restrição proteica (PB = 4,33%) por 14 dias até o momento do abate. De todos os parâmetros de qualidade de carne avaliados, houve diferença para força de cisalhamento Warner-Bratzler (WBSF) (P = 0,0055) e colágeno total (P = 0,0221), sugerindo que 14 dias de restrição de proteína aos animais afeta negativamente a maciez da carne e aumenta a quantidade de colágeno no músculo. Adicionalmente, foram realizadas análises de metabolômica em músculo esquelético e fígado, observando-se menor abundância dos aminoácidos valina (P = 0,0228), asparagina (P = 0,0149) para musculo e alanina (P = 0,0196), glutamato (P = 0,0144), aspartato (P = 0,0278) em fígado dos animais com restrição proteica. Para alguns aminoácidos utilizados como biomarcadores da degradação muscular, como tirosina e histidina, não diferiram (P > 0,05), o que sugere que a restrição proteica na dieta durante 14 dias não teve efeito sobre a degradação de proteína do tecido muscular.

ABSTRACT

RAMÍREZ, Germán Darío Zamudio, M.Sc., Universidade Federal de Viçosa, February, 2017. **Effect of protein restriction on diet on ewe's meat quality.** Adviser: Mário Luiz Chizzotti. Co-advisers: Cristina Mattos Veloso and Marcio de Souza Duarte.

It is known that calpains are the most important proteases that act in the regulation of synthesis and degradation of the muscle *in vivo*, as in the transformation of the muscle into meat, a fact that can be affected by the restriction of nutrients. Therefore, the objective of this study was to evaluate the effect of abrupt protein reduction in the diet on the organoleptic characteristics of the ewe's meat in the final stage of finishing in confinement. There were used 14 crossbred ewes (Dorper x Santa Ines), with average body weight of 39 $\pm$ 9.4 kg, which were confined in the finishing phase, during 46 days. At the beginning of the experiment, all animals were randomly organized into two groups, receiving the same diet for 32 days, containing 13.3% crude protein. On the 33rd experimental day a group (n = 7) was submitted to a diet with protein restriction (CP = 4.33%) for 14 days until the time of slaughter. Of all meat quality parameters evaluated, there were differences for Warner-Bratzler shear force (WBSF) (P = 0.0055) and total collagen (P = 0.0221), suggesting that 14 days of protein restriction to animals negatively affected Tenderness of the flesh and increases the amount of collagen in the muscle. In addition, metabolic analysis was performed on skeletal muscle and liver, with a lower abundance of amino acids valine (P = 0.0228), asparagine (P = 0.0149) for muscle and alanine (P = 0.0196), glutamate (P = 0.0144), aspartate (P = 0.0278) in the liver of animals with protein restriction. For some amino acids used as biomarkers of muscle degradation, such as tyrosine and histidine, they did not differ (P > 0.05), suggesting that protein restriction in the diet for 14 days had no effect on protein degradation of muscle tissue.

INTRODUÇÃO GERAL

A criação de ovinos, no Brasil, desenvolve-se, principalmente, em sistemas de produção extensivos, com regimes de alimentação inteiramente em pasto (Costa et al., 2008). Por conseguinte, estas condições favorecem que a produção de carne seja mais barata, devido ao uso dos recursos nutricionais provenientes das gramíneas ser de baixo custo (Tonetto et al., 2004; Detmann et al., 2010). No entanto, esses sistemas de produção são afetados pela sazonalidade ao longo do ano, sendo a estação seca, aquela na qual a qualidade da forragem perde em suas propriedades físicas e reduz seu valor nutricional (Detmann et al., 2004; Paulino, Detmann & Barros, 2008; Silva et al., 2009). Portanto, os níveis de proteína bruta inferiores a 70 g/kg de matéria seca na dieta de ruminantes prejudicam a capacidade fermentativa das bactérias sobre a fibra e, consequentemente, reduz o consumo de matéria seca e de outros nutrientes (Paulino, 1999; Paulino et al., 2002; Gotoh, 2012). Assim, para satisfazer as exigências do animal surgem alternativas como os sistemas de confinamento.

Atualmente, os sistemas de confinamento para ovinos têm despertado grande interesse entre os produtores das regiões Sudeste e Sul do Brasil, por reduzir a idade dos animais para abate, proporcionando mais carne ao longo do ano e retornando mais rápido o capital investido (Barros, Fernandes & von Linsingen, 2008; Medeiros et al., 2009). Outro aspecto que deve ser considerado nos sistemas de terminação em confinamento é a qualidade das carcaças e composição química dos tecidos musculares, uma vez que estes poderiam ser alterados de acordo com a quantidade e qualidade de proteína na dieta (Fluharty & MacClure, 1997; Zundt et al., 2001; Silva et al., 2002). No entanto, a proteína é um dos ingredientes mais caros numa ração, o que faz sua utilização um pouco limitada. Portanto, a otimização no uso deste ingrediente na dieta dos animais é essencial para estimular o desenvolvimento muscular (Montossi et al. 2013; Ponnampalam et al., 2016). Assim, quando o fornecimento de proteínas na dieta é alto, resulta em maior produção de amônia no trato gastrintestinal, que é absorvida e transportada para o fígado, para conversão em ureia e, finalmente, ser excretada quando em abundancia (Siddons et al., 1985). Consequentemente, o excesso de ureia é prejudicial

para o desempenho animal, devido à maior demanda do gasto energético para eliminação da mesma. Por outro lado, o fornecimento deste nutriente abaixo dos níveis requeridos para ruminantes afeta o equilíbrio de proteína/energia consumida, acarretando alterações na taxa de síntese e degradação de proteínas no tecido muscular e interferindo com as propriedades organolépticas da carne (Hernández et al., 2009; Ponnampalam et al., 2016).

Uma carne de boa qualidade deve atender os seguintes parâmetros: cor, textura, sabor, suculência e maciez (Joo et al., 2013; Holman et al., 2015; Ponnampalam et al., 2016). No entanto, a qualidade da carne é um atributo complexo e varia de acordo com as preferências pessoais de cada consumidor e cada nicho de mercado (Moreno, 2012). Segundo Osório et al. (2008), para o produtor, a qualidade deve ser remunerada; enquanto, para o consumidor, um produto de qualidade é aquele que resulta em um alto grau de satisfação. Portanto, a busca da satisfação do cliente deve ser constante e, por isso, deve haver um entendimento mútuo entre os segmentos. Em relação aos parâmetros de qualidade da carne ovina no Brasil a maciez é um dos principais atributos (Silva Sobrinho, 2001).

A maciez da carne é o resultado da atividade de vários complexos enzimáticos, tais como o sistema de calpaína/calpastatina, proteassomal e catepsinas (Therkildsen & Oksbjerg, 2009). No entanto, as calpaínas são enzimas também responsáveis pela conversão do músculo em carne (Rowe et al., 2004) e sobre elas se há focado várias pesquisas com o intuito de melhorar a maciez da carne, por meio da manipulação da atividade destas enzimas. Alguns trabalhos desenvolvidos por McDonagh et al. (1999), Kristensen et al. (2002, 2004), Therkildsen e Oksbjerg (2009) mostram que a manipulação destas enzimas por meio da dieta dos animais tem um impacto sobre o *turnover* do músculo *in vivo*. Outros trabalhos com restrição de nutrientes, como proteína e energia, por períodos curtos de tempo, indicam alteração no equilíbrio de síntese e degradação dos músculos, diminuindo a síntese e aumentando a degradação proteica (Millward & Waterlow 1978; Andersen et al., 2005). Por conseguinte, o objetivo com este trabalho foi avaliar o efeito da redução abrupta da proteína da dieta sobre as características organolépticas da carne e o perfil metabólico do músculo esquelético e fígado durante a fase final de acabamento em ovelhas.

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Article

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Effect of protein restriction on diet on ewe's meat quality

Summary

The restriction of nutrients in the diet of the animals could affect the rate of protein turnover in the skeletal muscle and the liver, as well as alterations in the tenderness of the meat. Therefore, a study was conducted using as a feeding strategy an abrupt reduction in the level of protein in the diet during the final phase of finishing. A total of 14 crossbred ewes (Dorper x Santa Ines) was randomized into two groups. The first group received a feed regimen that attended all nutritional requirements (CT = 13.3% crude protein, n = 7) throughout the experimental period and the second group had a reduced protein diet (LP = 4.33% crude protein, n = 7) 14 days before to slaughter. Of the meat quality traits evaluated, the results for Warner-Bratzler shear force (P = 0.0055) and total collagen (P = 0.0221) were higher for animals with restriction. In addition, metabolomics analysis was performed on skeletal muscle and liver tissues, with significant differences in amino acids such as valine (P = 0.0228), asparagine (P = 0.0149) for muscle and alanine (P = 0.01964), glutamate (P = 0.01443) and aspartate (P = 0.02787) in the liver, with lower abundance of animals with restriction. Our data suggest that 14 days of restriction negatively affect tenderness of the meat, possibly by increasing the amount of collagen.

Keywords: amino acids, meat, protein, tenderness.

1. Introduction

The regulation of skeletal muscle tissue in each living being depends on the contribution of nutrients, mainly amino acids responsible for protein synthesis. In ruminant animals, the microbial protein synthesized in the rumen is the main contributor of amino acids for muscle tissue growth. These animals require a minimum level of crude protein of about 70 g/kg of dry matter in the diet (Gotoh, 2012; Paulino, 1999). Therefore, a reduction in the protein level in the diet affects the fermentative efficiency of the ruminal bacteria and consequently the intakes of dry matter and energy by the animal (Paulino et al., 2002). A reduction in the level of protein in the diet causes a homeostasis challenge and an imbalance in skeletal muscle *turnover* (Du et al., 2004), altering the quality of the meat.

Turnover occurs in any type of body tissue, which is controlled by a balance between synthesis and degradation, producing protein deposition when there is increased synthesis and/or reduction of degradation rates (te Pas, Everts and Haagsman, 2004).

This physiological process is mediated by ribosomes for synthesis and controlled by enzymatic complexes such as calpain/calpastatin, lysosomal cathepsins and proteasome for the breakdown of muscle proteins (Therkildsen and Oksbjerg, 2009). Of all the enzymes mentioned above, calpains are those that actively acts on the *in vivo* degradation of the structural muscle protein, as well as being responsible for the transformation of the muscle into meat (Przybylski et al., 2015; Therkildsen and Oksbjerg, 2009; Du et al., 2004). Nutritional strategies have been proposed to stimulate muscle protein degradation and improve meat quality traits, mainly tenderness (Therkildsen and Oksbjerg, 2009; Kristensen et al., 2002, McDonagh et al., 1999).

It was hypothesized that the reduction in protein level in the diet increases muscle protein degradation to support the needs of nitrogen sources for the ruminal microbiota. Therefore, as objective of our study, we evaluated the effect of abrupt reduction of protein in the diet during the 14 days before to slaughter on the metabolic profile of muscle and liver and meat quality traits.

2. Material and methods

This study was conducted in the Department of Animal Science of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. All procedures were approved by the Committee of Animal Care and Use of the of the Universidade Federal de Viçosa, (protocol number 65/2016-CEUAP/UFV).

2.1. Animal handling and diets

Fourteen crossbred ewes (initial body weight = 39.3 $\pm$ 9.4 kg) were used, which were grouped into two treatments, consisting of two levels of crude protein in the diet. Both groups were fed the same crude protein content in the diet (CP = 13.3%) as proposed by the National Research Council of Nutrient Requirements for Small Ruminants (NRC, 2007). One group was fed a low crude protein diet (CP = 4.33%) for 14 days before slaughter, while the other group was fed same diet until slaughter. The chemical composition and proportion of ingredients of the experimental diets are presented in Table 1.

Table 1. Ingredients proportion and chemical composition of experimental diets

Control diet		Low protein diet	
Ingredient	**% dry matter**	**Ingredient**	**% dry matter**
Sugar cane	30.0	Sugar cane	30.0
Corn meal	55.4	Corn meal	35.0
Soybean meal	12.1	Cassava flour	28.0
Urea	0.50	Molasses	5.00
[1]Mineral mixture	2.00	[1]Mineral mixture	2.00
Chemical composition (%)		**Chemical composition (%)**	
Dry matter	69.9	Dry matter	69.5
Crude protein	13.3	Crude protein	4.33
Ether extract	2.41	Ether extract	1.66
Neutral detergent fiber	25.4	Neutral detergent fiber	22.9
Ash	4.33	Ash	4.93
Non-fibrous carbohydrates	54.5	Non-fibrous carbohydrates	66.1

[1]Mineral mixture: Ca = 140 g; P = 65 g; Mg = 10 g; S = 12 g; Na = 130 g; Co = 80 mg; Fe = 1000 mg; I = 60 mg; Mn = 3.000 mg; Se = 10 mg; Zn = 5.000 mg; F = (máx) 650 mg; Vit. A = 50.000 U.I.; Vit. E = 312 U.I.

2.2. Sampling and analysis of food, leftovers and feces

All feeding stuffs supplied to the animals were weighed daily. On the other hand, samples of each ingredient, food leftovers, feces and then dried in a forced ventilation oven at 55 °C for 72 hours were collected for further consumption calculations. All samples were analyzed for dry matter (DM), mineral matter (MM), crude protein (CP), ether extract (EE) per the procedures described by the Association of Official Analytical Chemists (AOAC, 1990). In terms of neutral detergent fiber (DNF) analyzes, were conducted per the recommendations of Mertens et al. (2002). For fecal collection, these were performed for three consecutive days, being weighed and removed every 24 hours. Of each day of collection, a representative sample was obtained and then placed in a forced ventilation oven at 55 °C for 72 hours for subsequent analysis of nitrogen concentration using the Kjeldahl method (INCT-CA N- 001/1; Detmann et al., 2012). All the analyzes described above were carried out in the Animal Nutrition Laboratory in the Animal Science Department of the Universidade Federal de Viçosa.

2.3. Sampling and analysis of blood, urine and ruminal fluid

For determination of plasma urea nitrogen (UN), blood samples were collected for three days at 48-hour intervals by direct puncture in the jugular vein and stored in vacuum tubes with separator gel (BD Vacutainer® SST II Advance). The blood samples were centrifuged at 3600 x g for 20 minutes to obtain the plasma and immediately analyzed by the enzyme-colorimetric method (InVitro®, UreiaLiquicolor), using an automatic device for biochemistry (Mindray®, model BS200E). In urine nitrogen analysis, were

estimated by the Kjeldahl method (INCT-CA N-001/1; Detmann et al., 2012), preceded by a total collection of urine for three consecutive days, using Individual cages coupled with funnels under the floor (Walker and Faichney, 1964) and silk meshes, for separation of urine and feces. During the test days, 100 ml of 20% H_2SO_4 were added to each collection vessel for urine preservation and at the end of each 24-hour period, the urine volume was measured and then a 10% sample of the Volume of each one daily and preserved at -20 °C for further analysis. In addition, the concentration of ammoniacal nitrogen in ruminal fluid was determined by the indophenol - catalyzed colorimetric reaction method (INCT-CA N-006/1; Detmann et al., 2012). For this purpose, ruminal fluid samples were collected during the slaughter of the animals, which were previously filtered in three layers of gauze and preserved in 50% H_2SO_4 at the rate of one ml for each 40 ml of fluid and then maintained at -20 °C for the corresponding analyzes.

2.4. Slaughtering, carcasses evaluation and muscle and liver sampling

The management of the animals during the slaughter was carried out in accordance with animal welfare practices, following the slaughter procedures of the Sanitary and Industrial Inspection Regulation for Animal Origin Products (Brasil, 1997). After slaughtering each animal, a sample of *Longissimus Lumborum* muscle (LL) and liver were immediately taken, which were pulverized in liquid nitrogen and stored in cryogenic tubes, containing two grams of each tissue for later analysis of metabolomics.

During slaughtering the carcasses and non-carcasses components were weighed for calculations of hot carcass weight and carcass performance. Afterwards, the carcasses were refrigerated at 4 °C for 24 h, at which temperature and pH measurements were taken, in the left side of LL as the anatomical measurement site at the caudal end on the union Sacro-lumbar, as described by Hopkins et al. (2011). Temperature and final pH were measured about 24 h *post mortem* in the same section of LL muscle, using a potentiometer with a glass probe for pH and temperature (SevenGo^{TM}, Mettler Toledo-Schwerzenbach, Switzerland).

Twenty-four hours after cooling carcasses, were weighed for calculations of cooling losses. Also, the subcutaneous fat thickness and ribeye area were measured at the 12th and 13th rib height. For the measurements of subcutaneous fat thickness, a precision caliper with millimeter graduation, placed at ¾ of ventral length on LL, was used (Greiner et al., 2003). Ribeye area were drawn on semi-transparent paper (Diniz et al.,

2016) and later analyzed using Image J software (National Institutes of Health, NIH USA). After all measurements on the carcasses, were collected from the right-side Longissimus muscle (Pannier et al., 2014). Each muscle sample was removed from the subcutaneous fat, epimysium and then sectioned into one-inch thick steaks before being vacuum packed and preserved at -20 °C for subsequent meat quality analysis (Silva et al., 2017).

2.5. Measurements of meat quality

From the one-inch steaks obtained from each *Longissimus* muscle, color, water losses, Warner-Bratzler shear force (WBSF), sarcomere length, myofibrillar fragmentation index (MFI) and collagen content were estimated. All the above analyzes were conducted at the Meat Science Laboratory of the Department of Animal Science of the Universidade Federal de Viçosa (LCC-UFV).

The meat color measurement was performed on a steak with 16 h after thawing at 4 °C and 30 minutes of rest outside of its packaging. This measure was assisted by a colorimetric instrument (HunterLab MiniScan EZ 45/0 LAV), taking five repetitions at different points of each steak. The measured variables were, *L **, *a **, *b **, Hue and Chroma according to the CIELab scale (MacDougal, 1994).

Thawing losses were estimated by weight difference between the steaks before and after thawing, the samples being thawed at 4 °C for 16 hours. The same steaks that were previously thawed, were packed in the vacuum and then placed in a water bath at 70 °C until reaching an internal temperature of the steak similar to that of the water (Christensen et al., 2000). Afterwards, the steaks were placed in an ice bath for 10 minutes and after that time, they were removed from their packaging to be weighed again, the gravimetric difference between the steak being thawed and cooked being the estimate of water losses by cooking (Li et al., 2012). The results of water losses by thawing and cooking were used to estimate the total losses of water of each steak, using the following equation:

Total water loss (%) = [(frozen steak weight – steak cooked weight) / frozen steak weight] * 100

WBSF was determined in steaks cooked in a water bath at 70 °C, using the same methodology described for estimation of water losses by cooking described by Christensen et al. (2000). after cooking the steaks, they were placed in refrigeration at 4 °C for 24 h. elapsed time of cooling, 5 samples of meat with cylindrical shape and 1.27

cm in diameter were collected, cut parallel to the muscle fibers, using a stainless-steel device for the extraction of samples (AMSA, 1995). Shear strength was determined by perpendicular incision of the muscle fibers of each cylinder of meat with a device support Warner-Bratzler shear (G-R Electrical Manufacturing Company, Manhattan, KS, USA).

For sarcomere length, *longissimus* muscle samples were collected at 24 h *post mortem* and small cubes, were excised in duplicate from each sample. The cubes were fixed in glutaraldehyde (5% glutaraldehyde in 0.1 M $NaHPO_4$ buffer at pH 7.2) for 4 hours at 4 °C and subsequently washed in 0.2 M sucrose solution buffered pH 7.2 as described by Koolmees, Koreknie and Smulders (1986). Sarcomere length was measured by laser diffraction using a 05-LHR-021 laser, (Melles Griot, Carlsbad, CA) and calculated as described by Cross, West, and Dutson (1981), using the following equation:

$$\text{Sarcomere length } (\mu m) = \frac{632.8 \times 10^{-3} \times D \times \sqrt{\left(T/D\right)^2 + 1}}{T}$$

where D equals the distance (mm) from the specimen-holding device to the screen (throughout this experiment, D had a constant value of 120mm) and T equals the separation (mm) between the zero and the first maximum band. six fibers were measured by everyone on each sample piece.

MFI were determined on fresh muscle as proposed by Olson, Parrish, and Stromer (1976) and modified by Culler, Parrish, Smith, and Cross (1978). The protein concentration of the myofibril suspension was determined by the Biuret method (Gornall, Bardawill, and David, 1949). Aliquots of the myofibril suspension were diluted with an isolating medium to reach a protein concentration of 0.5 ± 0.05 mg/ml. The diluted myofibril suspension was stirred and poured into a cuvette and the absorbance of this suspension was measured immediately at 540 nm. Absorbance was multiplied by 150 to give a MFI for each sample.

For determination of the collagen content, the method proposed by Woessner (1961), adjusted by Hadlich et al. (2006) and Latorre et al. (2016) was used. In brief, 1.5 g of lyophilized muscle was used in each of the 50 ml centrifuge tubes containing 12 ml of phosphate buffered saline (NaCl 137 mM, KCl 2,7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1,8 mM, pH 7.4). The tubes were placed in a water bath at 80 °C for 60 minutes with constant stirring. Subsequently, the tubes were transferred to a cold - water bath for 10 minutes. The supernatant fluids and the solid residues of the sample were separated by

centrifugation (6000 x g, 2 °C and 10 minutes). Then each fraction was hydrolyzed in 6N HCl at 110 °C for 16 h. After the hydrolysis, the samples were filtered through filter paper No. 2 (200 mg and 900 mg of activated carbon were added to the supernatant and residue respectively). The samples were then adjusted to pH 6.5-7.0 with 2N NaOH and filtered through No. 2 filter paper in a volumetric flask, diluting the supernatant in 200 ml and the residue in 250 ml of distilled water. For determination of the hydroxyproline content, 1 ml of filtrate was required. For each sample, 2 ml of Isopropanol (2-Propanol solution, Sigma Aldrich) and then 1 ml of Oxidant solution (1.41 g of chloramine T dissolved in 100 ml of buffer solution, 30 g of citric acid monohydrate, 15 g NaOH, 90 g of sodium acetate Trihydrate, dissolved in 500 ml of dH_2O). The tubes with the samples were rested for 4 minutes at room temperature and then 1 ml of Ehrlich reagent was added to each sample (2 g of P-dimethylbenzaldehyde, 2.5 ml of 70% Perchloric acid). The samples were vortexed, then covered with foil and placed in a water bath at 60 °C for 25 minutes. Subsequently, the tubes were transferred to a cold-water bath for 5 minutes. After that time, the samples were read by spectrophotometry at 558 nm, calculating the collagen content, multiplying the amount of hydroxyproline by 7.25 (Cross, Carpenter and Smith, 1973).

2.6. Metabolomic analysis

Metabolomic analyzes were performed per the procedure reported by Lisec et al. (2006). For this, 50 mg of each sample of M. LL and liver were weighed, which were collected during the slaughter and analyzed later through GC-MS. Prior to the processing of the samples, all material used for manipulation was cooled to avoid thawing of the biological sample. The procedures for preparation of samples prior to GC-MS analysis was performed in two steps (extraction and derivatization), which will be described below.

2.6.1. Extraction

50 mg of sample, previously pulverized in liquid nitrogen and stored at -80 °C in Eppendorf's tubes, were added 700 µl 100% Methanol and then vortexed for 10 seconds, to stop all enzymatic activity in the sample. 30 µl Ribitol stock (0.2 mg/ml milk, water) was added to each sample as an internal quantitative standard for the polar phase, and immediately the samples were vortexed for 10 seconds. The homogenized samples were shaken for 15 minutes at 70 °C and 1000 r.p.m. With the use of a thermomixer (after 2 minutes of incubation in the thermomixer, the tubes were opened

momentarily, to let out the extra pressure). After the samples were incubated, they were centrifuged for 10 minutes at 14000 r.p.m., to separate the supernatants, which were then transferred into new Eppendorf's tubes. To each supernatant, 375 µl of chloroform ($CHCl_3$) and 750 µl H_2O miliQ were added and carefully vortexed for 15 seconds. Finally, each supernatant was centrifuged for 15 minutes at 4000 r.p.m. and a 100 µl sample of the upper phase (polar phase) was taken, which was transferred to each new Eppendorf tube and immediately placed in Speed Vac without heating for 12 hours.

2.6.2. Derivatization

After drying in Speed Vac, samples were resuspended with 40 µl Methoxyaminhydrochlorid (20 mg/ml in Pyridin), shaken with thermomixer for two hours at 37 °C and 950 r.p.m. and then centrifuged for one minute at 4000 r.p.m. Afterwards, 70 µl MSTFA (1 ml + 20 µl FAME) were added and again agitated with thermomixer for 30 minutes at 37 °C and 950 r.p.m. Each sample incubated in the thermomixer was transferred into reading vials for GC-MS.

2.6.3. GC-MS TruTOF procedure

After derivatization, the samples were made using a GC-MS TruTOF system, using a chromatograph Agilent, Technologies 7890A and spectrophotometer TruTOF® HT TOFMS, Leco, equipped with a 30-m capillary column (DB-35 MS, Agilent Technologies), following the recommendations of Lisec et al. (2006). 1 µl of each sample was injected in *splitless* mode at 230 °C, charged by the helium gas, with continuous flow of 2 ml/min. Initially the temperature was maintained at 80 °C and then increased to 15 °C/min until reaching 330 °C, maintaining that temperature for 6 minutes. The chromatograms had a baseline and a deconvolution performed through the ChromaTOF software (LECO). Afterwards, the peaks were identified through the deconvoluted spectra obtained, using the TagSearch software (Cuadros-Inostroza et al., 2009). The areas of the chromatographic peaks of the fragmented ions were normalized by the peak area corresponding to ribitol and corrected for cell density (DO_{600nm}).

2.7. Statistical analysis

A completely randomized statistical design was used. The data were subjected to analysis of variance (ANOVA) to evaluate the effect of the nutritional plane on the qualitative characteristics of the meat. For all statistical procedures, was adopted $\alpha = 0.05$. All statistical procedures were performed with PROC GLM of SAS 9.0 statistical software (Statistical Analysis System Institute, Inc.).

The metabolomic data were analyzed using the web-based tool MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/; (Xia and Wishart, 2016)). The metabolite concentration table was uploaded to MetaboAnalyst and the data was log-transformed and Pareto-scaled before analysis. The T test was used to find the metabolites that differed significantly in muscle and liver concentrations between the protein restriction and control treatments ($P < 0.05$)

3. Results and discussion

3.1. Intake of nutrients

Protein restriction caused a decrease in the intake of DM and digestible energy (Table 2), therefore we considered that the animals also passed through an energy restriction. Hence, hereafter, the discussion of our replies will be made based on a restriction of both protein and energy in animals, because the symbiotic relationship between the microorganisms and the host does not allow the restriction of a single nutrient without affecting the others.

Table 2. Means of daily ingestion of dry matter, crude protein, digestible energy, neutral detergent fiber and neutral detergent fiber digestibility in ewes fed with and without protein decrease in the diet for 14 days before slaughter.

Items	Diet		SEM	P-Value
	Control	Low Protein		
Dry matter intake (kg/day)	1.38	1.06	0.1109	0.0186
Crude protein intake (kg/day)	0.18	0.04	0.0128	< 0.0001
Digestible energy intake (Mcal/day)	5.48	3.90	0.3691	0.0018
Neutral detergent fiber intake (Kg/day)	0.33	0.26	0.0219	0.0100
Digestibility of neutral detergent fiber (%)	51.3	43.8	0.0209	0.0054

Significant difference ($p < 0.05$).
SEM=Standard error mean.

3.2. Effect of protein restriction on serum urea nitrogen, and nitrogen content in urine, feces and ruminal content

The concentration of ruminal ammonia, as well as levels of urine nitrogen and fecal excretion of the animals with nutritional restriction of the present experiment, had significant differences ($P < 0.0001$), being lower in all variables evaluated in comparison to the control animals (Table 3). On the other hand, plasma urea nitrogen (UN), which is an indication of efficiency in the use of amino acids and whose concentration varies depending on the needs of the organism (Li et al., 2016). In our

case, the concentrations of the UN were lower for the animals with restriction (Table 3), which agrees with the results obtained by Li et al. (2016), Fernandez-Figares et al. (2007) and Mejia-Gudadarrama et al. (2002), who suggested that the low concentrations of the UN are possibly due to greater efficiency in the use of the N or to lower protein degradation. The low levels of ammonia in the rumen and UN in animals with restriction allow us to elucidate that 14 days of nutritional restriction had no effect on compensatory mechanisms to restore normal concentrations of ammonia in the rumen. It might be thought that an ammonia deficiency in the rumen could cause muscle tissue degradation as a compensatory effect, as was suggested in the hypothesis of our work. However, the concentration of UN is not a reliable parameter for evaluating proteolysis of muscle tissue, since there may also be degradation of other tissues. Therefore, the metabolomic is proposed as a specific analysis to determine muscle tissue degradation, which will be discussed later in this text.

Table 3. Means, plasma urea nitrogen, ruminal ammonia, urinary nitrogen and fecal nitrogen in ewes fed with and without protein reduction in the diet for 14 days before slaughter.

Items	Diet		SEM	P-Value
	Control	Low Protein		
Plasma urea-N (mg/100 ml)	31.3	3.53	1.3291	<0.0001
Ruminal ammonia (mg/100 ml)	9.70	0.79	0.3080	<0.0001
Urinary nitrogen (g/day)	19.3	1.99	0.0128	<0.0001
Fecal nitrogen (g/day)	5.68	2.91	0.3691	<0.0001

Significant difference ($p < 0.05$).
SEM=Standard error mean.

3.3. Carcass traits

The short-term effect of protein intake in ewes did not affect the characteristics of the carcasses, except for the subcutaneous fat thickness that was lower for ewes fed the restricted diet (Table 4). The reduction of the subcutaneous fat thickness is possibly due to a reduction in energy consumption as shown in table 2 of the present study. Consequently, the reduction in energy consumption leads to the use of endogenous sources as a compensatory mechanism (Eitam et al., 2012; Jenkins-Kruchten et al. 2003). The adipose tissue and the liver, constitute the main organs of supply of substrates for production of energy (Cunningham, 2003). Possibly, the lower thickness of subcutaneous fat observed in animals with restriction could be a consequence of a greater mobilization of fatty acids from the adipose tissue, or a greater retention of volatile fatty acids in the circulation porta-hepatic, coming from the rumen (Eitam et al.,

2012; Cunningham, 2003). On the other hand, the low energy content consumed, reduce the muscle glycogen levels and consequently affect the final pH of the muscle (Vestergaard et al., 2000). However, in our study no differences (P = 0.5201) were observed in the final pH of the carcasses (Table 4). Of the above, it would be expected that the final pH of the carcasses of the animals with restriction would be higher, due to lower energy consumption.

Table 4. Carcass traits of ewes fed with and without protein reduction in the diet.

Items	Diet		SEM	P-Value
	Control	Low Protein		
Initial body weight (kg)	38.8	39.8	3.6951	0.8563
Final body weight (kg)	48.5	45.2	3.2950	0.4933
Empty body weight (kg)	42.3	39.4	3.1161	0.5250
Average daily gain (g/d)	209	116	26.244	0.0282
Hot carcass weight (kg)	23.8	21.8	1.8676	0.4729
Hot carcass yield (%)	48.9	48.1	0.7619	0.4233
Cold carcass weight (kg)	23.2	21.3	1.8083	0.4573
Cold carcass yield (%)	47.8	46.8	0.7509	0.3532
Viscera from non-carcass components (kg)	18.5	17.6	1.2896	0.6202
Liver (kg)	0.79	0.70	0.0676	0.3583
Gastrointestinal tract (kg)	2.67	3.02	0.1573	0.1403
Ribeye area (cm^2)	16.6	15.3	1.0978	0.4203
Subcutaneous fat thickness (mm)	5.10	3.19	0.5975	0.0436
$Temperature_{24h}$ (°C)	6.93	6.47	0.3354	0.3542
pH_{24h}	5.45	5.47	0.0229	0.5201

Means in the same line followed by same letter do not differ (P > 0.05).
SEM=Standard error mean.

3.4. Meat quality traits

The protein restriction in the diet during 14 days, as well as its effects on the energy levels ingested, did not affect the qualitative characteristics of the meat (P > 0.05), except for BWSF and total collagen (Table 5). It was expected that the decrease in energy consumption (Table 2), had alterations in color components (Table 5), due to possible modifications in the metabolism of muscle fibers, from a glycolytic to an oxidative metabolism (Ponnampalam et al. 2016; Strydom et al., 2015; Lindahl, 2011; Du et al., 2009; Young and West, 2001; Ahn, et al., 1998; Renerre, 1990). On the other hand, the final pH of the carcasses (Table 4), which was not affected by the decrease in protein and energy consumption, could explain the lack of influence of color, as well as losses of water in the meat of the present study (Velasco et al., 2004, Diaz et al., 2002).

Table 5. Meat quality traits of ewes fed with and without protein reduction in the diet.

Items	Diet		SEM	p- value
	Control	Low Protein		
Thawing losses (%)	2.35	2.23	0.4370	0.7339
Cooking losses (%)	20.8	21.5	1.1935	0.4761
Total losses (%)	22.7	23.2	1.2564	0.5643
WBSF (N)	29.7	36.0	0.1551	0.0055
Sarcomere length (μm)	1.72	1.70	0.0470	0.7352
MFI	62.3	63.6	5.1086	0.8482
Total collagen (mg/g)	2.05	2.35	0.1522	0.0221
soluble collagen (%)	22.0	22.8	1.2988	0.4838
Colour *L**	39.75	38.29	1.0091	0.0847
Colour *a**	11.91	12.13	0.2727	0.3202
Colour *b**	12.87	12.60	0.3366	0.3295
Hue	47.37	46.34	0.7014	0.0755
Chroma	17.63	17.58	0.3787	0.8881

p-value < 0,05.
SEM=Standard error mean.

The WBSF indirect method used to determine tenderness of meat had a significant difference (P = 0.0055), with more shear resistance for meat of ewes with nutrient restriction (Table 5). There are several factors that influence the tenderness of the meat such as, amount of connective tissue, muscle fiber type, *post-mortem* enzyme activity, etc. (Starkey et al., 2016; Ramos and Gomide, 2007; Young, Hopkins and Pethick, 2005; Safari, Channon, Hopkins, Hall and van de Ven, 2002). Therefore, additional analyzes such as MFI, sarcomere length, quantification and solubility of collagen were performed (Ramos and Gomide, 2007). The MFI that is related to tenderness of the meat in a positive way and that determines the proteolysis of myofibrils post mortem (Koohmaraie et al., 2002). No difference was observed (P = 0.8482) for MFI, suggesting that 14 days of nutrient restriction in our animals, was little time to increase muscle protein degradation activity. Additionally, analyzes were performed for WBSF and MFI in meats with seven and fourteen post-slaughter days (Figure 1). The results showed no interaction between treatment and meat aging time for WBSF (P = 0.4555) and MFI (P = 0.4683). However, WBFS and MFI show a similar distribution pattern in both treatments at different post-slaughter times (Figure 1).

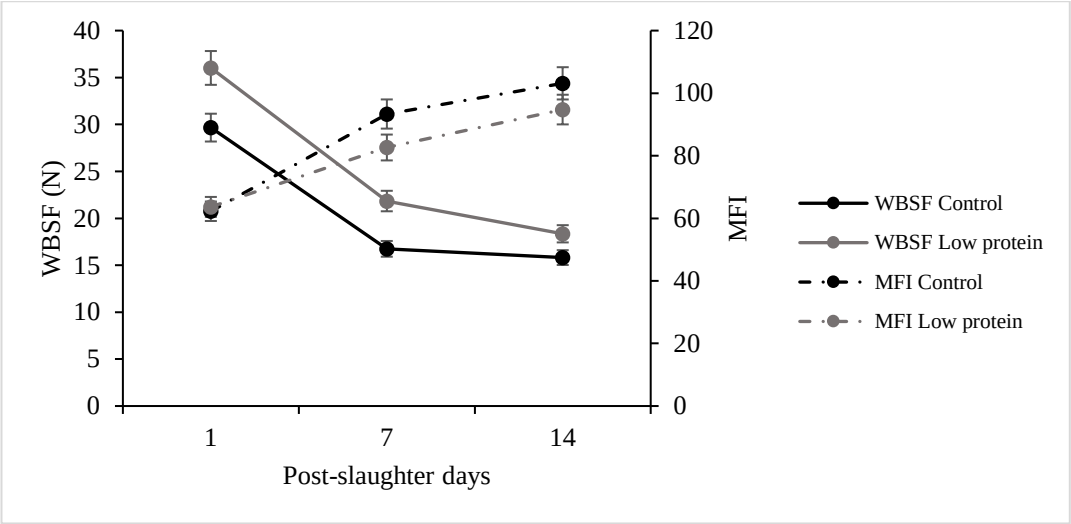

Figure 1. WBFS and MFI in meat of 1, 7 and 14 post-slaughter days of ewes fed with and without protein reduction in the diet. The bars represent the standard error of the means.

Finally, in the measurements of total and soluble collagen, differences were only found in total collagen, which was more abundant in the muscles of animals with restriction (P = 0.0221). From this result, we can infer that the hardness in the meat of animals with restriction was affected by the collagen content. However, some authors such as Warner et al. (2010), suggest that the total collagen content is limited to predicting the degree of tenderness in the meat. On the other hand, Young and Braggins (1993), concluded in this case that the total collagen concentration was the best indicator to measure tenderness in the meat of animals that are reaching their state of maturity. Similarly, Zhao et al. (2015), studying the deposition of collagen in lamb muscle; Observed greater activity in the signaling pathway p38 MAPK (p38 mitogen-activated protein kinases), responsible for the increase in collagen content in animals with nutritional restriction and consequently, increase in BWSF in muscle. As was observed in the previous study on the deposition pathways of collagen, it is recommended to carry out more studies related to this subject, since the analyzes in terms of quantity and solubility of collagen does not give us a deep explanation of the effect Which has on the tenderness in the meat.

3.5. Metabolic profile in muscle and liver in ewes fed two different diets

For muscle and liver, 191 and 199 metabolites, respectively, were identified of which the amino acids were detailed. The muscle amino acid profile of the animals with restriction had a few differences, except for the amino acids valine and asparagine, which were less abundant (Table 6). This result coincides with a study carried out by Palma et al. (2016), with nutritional restriction in different breeds of sheep, who found lower levels of the valine and related it to lower synthesis of muscle protein. On the

other hand, Li et al. (2016) found lower levels of the valine and higher concentration of asparagine in the muscle of pigs with protein restriction in the diet for 10 and 25 days. These authors suggested that the limitation of some essential amino acids such as valine in the muscle, prevent the synthesis of proteins and consequently, the levels of other non-essential amino acids like asparagine could increase in their concentration. According to Nelson and Cox (2009), valine and asparagine are glycogenic amino acid donors of carbon skeletons for the use in the citric acid cycle. Therefore, for our work, we suggest that the lower abundance of these amino acids in free form in the muscle, could be a consequence of their use of energy production, since these animals despite having been restricted with protein, were also affected in the consumption of energy. Other amino acids such as tyrosine and valine are used as indicators of muscle breakdown (te Pas, Everts and Haagsman, 2004). In our case, we observed that for these amino acids there were no differences in their concentrations between treatments (Table 6). Therefore, we suggest that 14 days of nutrient restriction have no effect on muscle protein degradation. On the other hand, histidine, which is used as a biomarker in the processes of degradation of myofibrillar protein in several species, being a constituent of the chains of actin and myosin (D'mello, 2003). In our case, the concentrations of free amino acid in the muscle were not affected with 14 days of nutrient restriction, contrary to what we were expecting. However, we should be cautious in giving this kind of interpretation, since in sheep this result is underestimated when measured in urine (Gopinath & Kitts, 1984). But, this result may be valid for us, because its measurement was performed directly on muscles, being quantified the free form of this compound.

Table 6. Metabolites that differed in ewe′s muscle extract samples with control and low protein diets.

Metabolite	Diet		p-value	-Log10(p)
	Control	Low Protein		
Amino acid				
Valine	0.018	0.012	0.0228	1.6416
Asparagine	0.354	0.183	0.0149	1.8264
Alanine	0.639	0.541	0.5936	0.2306
Histidine	0.011	0.015	0.3176	0.4288
Tyrosine	1.133	1.111	0.2593	0.8354
Glycine	0.937	1.295	0.2019	0.6948
Isoleucine	1.046	0.846	0.1063	0.9736
Leucine	1.061	0.906	0.2497	0.6264
Lysine	0.123	0.137	0.3829	0.2666
Methionine	1.070	1.011	0.7832	0.1061

Significant difference ($p < 0.05$).

Aspartate and glutamate, are glycogenic amino acids used to produce energy in the citric acid cycle (Nelson and Cox, 2009). As well as the donors of ammonia for the urea cycle (Newsholme et al., 2003). In our case the levels of aspartate and glutamate in liver were less abundant in animals with restriction (Table 7). Therefore, the reduced levels of these two amino acids are possibly a consequence of lower degradation, which can be corroborated by lower concentrations of serum urea nitrogen found in restricted animals (Table 3). On the other hand, alanine, which was also less abundant in liver of the restricted animals, coincides with the results found by Palma et al. (2016) in lamb with nutritional restriction and attributed its low concentration to lower levels of glucose found in animals. Also, McCue (2010) reports that alanine is one of the major contributors to gluconeogenesis. Therefore, we suggest that the free form of this amino acid in the liver of the animals with a restriction in the present study, may be diminished as a consequence of its use to obtain energy.

Table 7. Metabolites that differed in ewe's liver extract samples with control and low protein diets.

Metabolite	Diet		p-value	-Log10(p)
	Control	Low Protein		
Amino acid				
Alanine	0.028	0.015	0.0196	1.7068
Glutamate	0.020	0.018	0.0144	1.8407
Aspartate	0.028	0.014	0.0278	1.5549
Asparagine	0.031	0.027	0.3174	0.4983
Glutamine	0.021	0.020	0.2550	0.5934
Isoleucine	0.030	0.023	0.3001	0.5227
Leucine	0.037	0.036	0.2549	0.5935
Lysine	0.020	0.026	0.0710	1.1487
Methionine	0.025	0.019	0.2537	0.5956
Tyrosine	0.030	0.026	0.9297	0.0316
Valine	0.023	0.020	0.2128	0.6719

Significant difference ($p < 0.05$).

4. Conclusions

Fourteen days of nutritional restriction prior to slaughter in ewes, there was little time to increase muscle protein degradation. Therefore, the increased resistance to cuts in meat in animals with nutritional restriction, is due to an increase in the collagen content. In terms of the other meat quality traits evaluated, were not affected by the nutrient restriction.

The amino acid profile in muscle and liver showed few changes with a nutrient restriction during 14 days. However, the amino acids, valine, asparagine, glutamate, aspartate and alanine that were different in their concentration are due to a response to an energy restriction and not to a protein restriction.

Conflicts of interest

The authors have no conflict of interest to declare.

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