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Serum biochemical panel and modulation of skeletal muscle fibres: effect on the meat quality from lambs fed with different levels of whole cottonseed

Painel bioquímico sérico e modulação de fibras musculares esqueléticas: efeito na qualidade da carne de cordeiros alimentados com níveis distintos de caroço de algodão

Panel bioquímico sérico y modulación de fibras musculares esqueléticas: efecto sobre la calidad de la carne de corderos alimentados con diferentes niveles de semilla de algodón entera

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Abstract

The use of whole cottonseed (WCS) in the diet of ruminants has come to prominence mainly because this ingredient contains high levels of protein and lipids. The aims of this study were to evaluate serum biochemical panel and modulation of the skeletal muscle fibres (*Longissimus thoracis*) of Ile de France lambs fed with levels of WCS; and relationship between skeletal muscle fibres and meat quality. Fifty lambs were used in the experiment: five (5) reference and 45 equally distributed in treatments 0, 10, 20, 30 and 40% WCS (64 days feedlot time), using a completely randomised design. Blood biochemical determinations were performed using colorimetric kits. The fibers were determined by histochemistry method and classified as Type I, Type IIA, Type IIB and Type IIC. Muscle fiber characteristics were correlated with meat quality, considering variables of color, texture and sensory profile, physicochemical and fatty acid profile. Cholesterol levels, total protein and gamma-glutamyl transferase increased ($p \leq 0.05$) in line with increased levels of WCS in the diet. Muscle fibres modulation ($p \leq 0.05$) was observed, with an increase in red, a reduction in glycolytic, and modulation of Type IIB to Type IIA. The addition of WCS to the diet

increased ($p \leq 0.05$) the relative area of Type IIC. There was correlation between muscle fibres and meat qualitative characteristics. Time effect was more pronounced than diet effect on the muscle fibres modulation. Muscle fibres modulation was closely linked with meat quality.
Keywords: Histochemistry; Myosin ATPase; Meat quality; *Longissimus thoracis*.

Resumo

A utilização de caroço de algodão (CA) na dieta de ruminantes tem ganhado destaque principalmente por este ingrediente conter altos teores de proteínas e lipídios. Objetivou-se avaliar o painel bioquímico sérico e a modulação das fibras musculares esqueléticas (*Longissimus thoracis*) de cordeiros Ile de France alimentados com níveis de CA; e a relação entre fibras musculares esqueléticas e qualidade da carne. Foram utilizados 50 cordeiros: cinco (5) referência e 45 igualmente distribuídos nos tratamentos 0, 10, 20, 30 e 40% CA (64 dias de confinamento), em delineamento inteiramente casualizado. As análises bioquímicas no sangue foram realizadas utilizando kits colorimétricos. As fibras foram determinadas pelo método histoquímico e classificadas como Tipo I, Tipo IIA, Tipo IIB e Tipo IIC. As características das fibras musculares foram correlacionadas com a qualidade da carne, considerando variáveis de cor, perfis de textura e sensorial, físico-química e perfil de ácidos graxos. Os teores de colesterol, proteína total e gama-glutamil transferase aumentaram ($p \leq 0,05$) com o incremento de CA na dieta. Verificou-se modulação das fibras musculares ($p \leq 0,05$), com aumento das vermelhas, redução das glicolíticas e modulação do Tipo IIB para Tipo IIA. A inclusão de CA na dieta aumentou ($p \leq 0,05$) a área relativa do Tipo IIC. Houve correlação entre fibras musculares e características qualitativas da carne. O efeito de tempo foi mais pronunciado que o efeito de dieta na modulação das fibras musculares. A modulação das fibras musculares esteve intimamente ligada à qualidade da carne.
Palavras-chave: Histoquímica; ATPase miosínica; Qualidade da carne; *Longissimus thoracis*.

Resumen

El uso de la semilla de algodón entera (SAE) en la dieta de los rumiantes se ha destacado principalmente porque este ingrediente contiene altos niveles de proteínas y lípidos. Los objetivos de este estudio fueron evaluar el panel bioquímico sérico y la modulación de las fibras del músculo esquelético (*Longissimus thoracis*) de corderos Ile de France alimentados con niveles de SAE; y relación entre las fibras musculares esqueléticas y la calidad de la carne. En el experimento se utilizaron 50 corderos: cinco (5) de referencia y 45 distribuidos equitativamente en los tratamientos 0, 10, 20, 30 y 40% SAE (tiempo de engorde de 64 días), utilizando un diseño completamente al azar. Las determinaciones bioquímicas sanguíneas se realizaron utilizando kits colorimétricos. Las fibras se determinaron por método histoquímico y se clasificaron en Tipo I, Tipo IIA, Tipo IIB y Tipo IIC. Las características de la fibra muscular se correlacionaron con la calidad de la carne, considerando variables de color, textura y perfil sensorial, fisicoquímico y perfil de ácidos grasos. Los niveles de colesterol, proteína total y gamma-glutamil transferasa aumentaron ($p \leq 0.05$) en consonancia con el aumento de los niveles de SAE en la dieta. Se observó modulación de las fibras musculares ($p \leq 0.05$), con aumento de rojo, reducción de glucolíticos y modulación de Tipo IIB a Tipo IIA. La adición de SAE a la dieta incrementó ($p \leq 0.05$) el área relativa de Tipo IIC. Hubo correlación entre las fibras musculares y las características cualitativas de la carne. El efecto del tiempo fue más pronunciado que el efecto de la dieta en la modulación de las fibras musculares. La modulación de las fibras musculares estuvo estrechamente relacionada con la calidad de la carne.
Palabras clave: Histoquímica; Miosina ATPasa; Calidad de la carne; *Longissimus thoracis*.

1. Introduction

Animal diets involve high production costs and it is necessary to study alternative ingredients that can provide adequate nutrients at low cost. The use of whole cottonseed (WCS) in the diet of ruminants has come to prominence mainly because this ingredient contains high levels of protein (23%) and lipids (17.8%) (NRC, 2007). However, whole cottonseed can contain variable amounts of gossypol, a yellow pigment contained in cotton glands (Gadelha et al., 2014), which, in its free form, can be toxic to young ruminants when the detoxification capacity is exceeded (Solaiman et al., 2009). The fragility of erythrocytes has been used as an indicator of the toxicity of gossypol (Dayani et al., 2011; Câmara et al., 2016), whereas the analysis of serum biochemical panel reflects the nutritional status of animals (González et al., 2000).

The use of cotton by-products (seed, cake and meal) in the nutrition of small ruminants has been extensively researched, analysing its effects on production costs, ingestive behaviour, serum biochemical panel and carcass performance (Dayani et al., 2011; Lima Júnior et al., 2012; Piona et al., 2012; Rufino Junior et al. 2015; Pilecco 2018). The use of cotton by-products in the nutrition of small ruminants has also been studied in relation to its effect on the quality of meat, principally regarding factors such as the physicochemical composition, fatty acid and amino acid profile, the identification of volatile organic compounds,

and the sensorial acceptance of meat from lambs fed with cotton by-products (Madruga et al., 2008; Paim et al., 2010; Vieira et al., 2010; Paim et al., 2014; Viana et al., 2014; Pellegrini et al., 2020). However, there are no existing studies regarding the effect of the inclusion of cotton by-products in the diet of lambs on the skeletal muscle fibres, which is a relevant subject to be investigated.

Each muscle is formed by a combination of muscle fibres; the frequency of each type of fibre, its diameter and area, as well as its contractile and metabolic properties, are all important factors because they can directly influence the quality of meat, as well as promoting changes during the production of meat products (Ithurralde et al., 2015). Muscle fibres can be classified according to their metabolic, physiological and contractile properties. Direct comparisons between these properties have resulted in the classification of muscle fibres into the following three categories: Type I (red, aerobic, slow oxidative); Type IIA (intermediate, fast oxidative and glycolytic); and Type IIB (white, anaerobic, fast glycolytic) (Ryu & Kim, 2005; Chriki et al., 2012; Choe & Kim, 2014). The identification of muscle fibres (I, IIA and IIB) can be performed by using histochemical techniques, one of which is m-ATPase (myosin ATPase) (Brooke & Kaiser, 1970). The principle of this technique is based on the differentiated behaviour of muscle fibres at different pH values, which is due to the muscular concentration of alkaline or acid phosphatase (Loughlin, 1993).

The histochemical technique can detect another type of fibre, known as Type IIC (Peinado et al., 2004), which is considered to be a non-differentiated fibre or a precursor of Type IIA and Type IB fibres (Brooke & Kaiser, 1970); fibre that has undergone changes due to diseases or injuries (Ramos & Gomide, 2009); or immature fibre (Dubowitz et al., 2013). Type IIC fibres are considered to be hybrid fibres due to the coexistence of the heavy chain myosins I and II (mainly MHCIIa) (Hori et al., 1998); they have fast contraction speed and high fatigue resistance (Nakatani et al., 2003).

The quality of meat is a result of the characteristics of its muscular fibres, which can be influenced by factors such as differences between breeds (Bünger et al., 2009), age (Peinado et al., 2004) and muscle type (Ithurralde et al., 2015), as well as the food management and termination system used (Gallo et al., 2009; Santello et al., 2009; Santello et al., 2010; Joo et al., 2013; Hamdi et al., 2016).The aims of this study were to 1) to evaluate serum biochemical panel; 2) to evaluate the modulation of the skeletal muscle fibres (*Longissimus thoracis*) of Ile de France lambs fed with levels of WCS (0, 10, 20, 30 and 40%); and 3) to assess the relationship between the skeletal muscle fibres and the quality of the meat.

2. Methodology

This study received prior approval from the Animal Ethics Committee (CEUA) of the Farroupilha Federal Institute, Júlio de Castilhos, RS, Brazil, Protocol No. 01.0378.2015/001.2015.

2.1 Location

The experiments with the lambs were carried out at the Farroupilha Federal Institute of Education, Science and Technology, located in the city of Júlio de Castilhos, RS, Brazil. The municipality is located at 513 meters of altitude, latitude of 29º18'35"south, and longitude of 53º71'23" west.

2.2 Treatments

Fifty, uncastrated, Ile de France lambs were used in the experiments. Five of the animals were used as reference (slaughtered at the start of the experiment, 60 days old, average weight 20 kg); the other 45 lambs were confined and divided into five treatments with nine replicates of each. The treatments were based on different levels of whole cottonseed (*Gossypium hirsutum* L.) included in the diet (0, 10, 20, 30 and 40% WCS in dry matter); each gram of WCS contained 3.65 mg of gossypol

(AOCS, 1964). The diets were calculated in order to meet the nutritional requirements of growing lambs (NRC, 2007) and are shown in Table 1. After weaning (60 days) and adaptation to the experimental conditions (7 days), the lambs were finished in confinement, in fully covered, individual bays with a slatted floor, with approximately 2 m² area and equipped with drinking fountains and troughs. The diet was provided to the animals as total mixed ration (TMR), and finely ground WCS was incorporated into feed to avoid selection. Feeding was *ad libitum* and consumption was monitored twice a day at the pre-set times of 7:00 am and 4:00 p.m. After adaptation (initial mean weight 19.26 kg, age 67 days), the feeding trial was extended until lambs reached 36 kg live weight at slaughter, which corresponded to 60% of the live weight at maturity (64 days feedlot time, mean final weight 36.64 kg, age 124 days). The lambs were submitted to fasting from solids for 12 hours and then weighed. The animals were slaughtered using a pneumatic gun for desensitisation, which was followed by bleeding, skinning, evisceration, weighing, washing and cooling of the carcasses in a cold room at 2 °C for 24 hours (Brasil, 1952).

Table 1. Proportion of ingredients and chemical composition in the experimental diets, expressed as g kg^{-1} on a dry matter basis.

	Diets (whole cottonseed levels, %)				
	0	10	20	30	40
Ingredients					
Corn silage	400	400	400	400	400
Whole cottonseed	0	100	200	300	400
Corn meal	325.4	260.2	195.2	129.2	65.5
Soybean meal	250	215	180	146	110
Limestone	19.6	19.2	19.2	19.2	19.5
Mineral mixture[1]	5.0	5.6	5.6	5.6	5.0
Composition					
Dry matter (DM), as feed basis	626	629	633	637	641
Crude protein (CP)	180	180	180	181	181
Ashes	70	71	71	71	71
- calcium	10	10	10	10	10
- phosphorus	4.3	4.5	4.7	4.9	5.0
Acid detergent fiber (ADF)	143	171	198	226	253
Neutral detergent fiber (NDF)	286	320	354	388	422
Crude fat (CF)	27.6	45.9	64.2	82.5	101.0
Total digestible nutrients (TDN)	684	740	741	742	744
Digestible energy (DE), Mcal kg^{-1}	3.02	3.26	3.27	3.27	3.28
Metabolizable energy (ME), Mcal kg^{-1}	2.47	2.68	2.68	2.68	2.69

[1] Composition by kg: 152g of Ca; 85g of P; 135g of Na; 10g of Mg; 18g of S; 80mg of I; 1,400mg of Mn; 150mg of Mo; 25mg of Se; 60mg of Co; 4,000mg of Zn; 850mg of F. Source: Authors.

2.3 Blood sampling and serum biochemical panel

Prior to desensitisation, blood samples were collected by puncturing the jugular vein; the samples were centrifuged at 3,000 rpm to obtain the serum, which was stored at -12 °C until the time of analysis. In each treatment, the degree of hemolysis (%) was determined by the ratio of the number of hemolysed samples, by the total number of samples; the result was multiplied by 100. The serum activities of albumin (bromcresol green), cholesterol (enzymatic-Trinder), alkaline phosphatase (Roy), gamma-glutamyl transferase (GGT) (Szasz), glucose (GOD-Trinder), total proteins (Biuret), aspartate aminotransferase (AST) (Reitman and Frankel), alanine aminotransferase (ALT) (Reitman and Frankel) and triglycerides (enzymatic-Trinder) were determined using commercial colorimetric kits according to the manufacturer's instructions (Labtest Diagnóstica S.A, Lagoa Santa, MG, Brazil), using spectrophotometer (Cirrus 80 ST).

2.4 Muscle sampling

After the carcasses were cooled (24 h, 2 ºC), samples (1 cm x 1 cm x 0.5 cm) of the *Longissimus thoracis* muscle (right side, medial portion) were collected; the samples were stored in 2 mL centrifuge microtubes, frozen in liquid nitrogen, and kept at -80 °C until analysis (Ramos & Gomide, 2009).

2.5 Histochemistry, fibre types and morphometric analysis

The frozen muscle tissue samples were fixed on specific metal supports using optimal critical temperature compound (OCT Tissue-Tek®) and maintained (-20 °C) in Leica® CM1850™ cryostat equipment (Leica Microsystems, Wetzlar, Germany) until the moment of cutting. Serial cross-section cuts, 10 μm thick, were obtained to assemble the histological slides, which were adhered to the slides by approximation.

The histological sections were initially stabilised at room temperature (20 °C). They were then stained according to the protocol described by Loughlin (1993), which is based on variations in sensitivity for myofibrillar adenosine triphosphatase activity (mATPase or myosin ATPase) on exposure to different pH values. Two slides were prepared per experimental unit (animal), which were incubated in pH solutions of 4.3 and pH 4.6 in order to distinguish the different types of fibres.

The images were captured using an Olympus® optical microscope (model BX51, Olympus, Tokyo, Japan) with a 20X objective. Each slide captured the images of 20 microscopic fields, of which the ten best were chosen to analyse the fibres.

Depending on the intensity of their histochemical dyeing, when incubated at a given pH, the fibres presented pale, intermediate and dark colouration. The identification and classification of the muscle fibres was performed using the following classification: Type I (dark, pH 4.3); Type IIA (pale, pH 4.6); Type IIB (intermediate, pH 4.6); and Type IIC (intermediate, pH 4.3) (Loughlin, 1993).

For each type of fibre, the cross-sectional area (μm^2) and the minimun diameter (μm) were measured to avoid possible error because of tilted sections, using a grid mask and Image J (National Institutes of Health, NIH, Maryland, USA) software. The frequency (%) of the fibres was calculated by the ratio between the absolute number of each type of fibre in the grid mask and the total number of fibres that were counted, multiplying the quotient by 100 (Ramos & Gomide, 2009). The relative area (%) was determined by the ratio of the product of each fibre to its respective frequency, by the sum of the products of all the fibres by their frequencies; the result was multiplied by 100.

The contraction velocity, colour and metabolism of the fibres were expressed as the % of the relative area, and the types of fibres were classified as follows: slow (Type I), rapid (Type IIA + Type IIB + Type IIC), red (Type I + Type IIA + Type IIC), white (Type IIB); oxidative (Type I), oxidative-glycolytic (Type IIA + Type IIC), and glycolytic (Type IIB).

2.6 Data regarding quality of meat

In order to analyse the correlation between the characteristics of the skeletal muscle fibres and the quality of the meat the classification of Pellegrini et al. (2020) was used, which was as follows: colour (10 variables), texture and sensory profile (17 variables), physicochemical (13 variables) and fatty acid profile (18 variables).

2.7 Experimental design and statistical analysis

The data were subjected to outlier investigation from the studentized residual. They were subsequently submitted to univariate analysis of variance (ANOVA).

In order to verify the modulation of the skeletal muscle fibres during the confinement and termination phase, the data regarding the lambs slaughtered at the beginning of the experiment (reference) were analysed in conjunction with the data regarding the lambs slaughtered at the end of the experiment (confined). This was carried out using a completely randomised design with six treatments (reference, 0, 10, 20, 30 and 40% WCS) and a different number of replicates (five references, nine confined) using the GLM procedure. The means were adjusted by the ordinary least squares method using the LSMEANS command and were compared by contrast.

To evaluate the effect of the inclusion of WCS in the diet on the variables of serum biochemical panel and characteristics of skeletal muscle fibres, data regarding the lambs slaughtered at the end of the experiment were analysed in a completely

randomised design with five treatments (0, 10, 20, 30 and 40% WCS) and nine replicates of each; the initial weight of the animals was used as a covariate, using the GLM procedure. The means were adjusted using the ordinary least squares method with the LSMEANS command and compared by the t-test. In addition, the linear and quadratic trends were tested by contrasts derived from the coefficients for interpolation of the orthogonal polynomials. Furthermore, the polynomial regression was adjusted using the RSREG procedure and the r^2 values were expressed in relation to the source treatments (regression + lack of fit). Partial Spearman correlation analysis was then performed using the characteristics of the skeletal muscle fibres of *Longissimus thoracis* and 58 variables regarding meat quality, with the levels of WCS used as covariate.

The statistical analyses were performed using SAS® (Statistical Analysis System) software, version 9.4 (SAS Institute Inc., Cary, NC, USA) at 5% significance level.

3. Results and Discussion

3.1 Serum biochemical panel

As the level of whole cottonseed in the diet increased there was an increase in the degree of hemolysis of 0, 11, 22, 33 and 44% for the treatments with diets 0, 10, 20, 30 and 40% WCS, respectively ($\hat{y}_{hemolysis} = 1.1WCS$; $r^2 = 0.99$). Increased hemolysis is associated with biochemical changes induced by free gossypol that escapes the detoxification of rumen and/or bound gossypol, which was hydrolysed in the rumen and absorbed through the small intestine, causing erythrocyte fragility (Rogers et al., 2002).

There was no effect ($p>0.05$) due to the whole cottonseed on the glucose, triglycerides and albumin (Table 2). However, there was effect ($p\leq0.05$) due to the whole cottonseed levels on cholesterol and total protein. These changes were due to the fact that WCS is rich in proteins (23.3%) and lipids (22.7%) (Pilecco, 2018); the metabolism of these constituents directly influences their plasmatic concentrations. The inclusion of whole cottonseed in the diet increases the export of lipoprotein cholesterol through the gut, which is the main site for the synthesis of new cholesterol in ruminants (Noble, 1981). Previous studies have reported that cottonseed oil can raise the blood levels of cholesterol and triglycerides (Mata-Hernandez et al., 1978; Garcia et al., 2003). The increase in serum cholesterol levels can also be explained by the increase in oleic acid (18:1 cis-9), which is the main fatty acid precursor of cholesterol in mammals (Faria et al., 2012), given that, with increasing levels of whole cottonseed, the concentration of oleic acid in the diet increased (Pellegrini et al., 2020). The values found for the blood constituents were in agreement with the reference values for sheep (Feldman et al., 2000), and were corroborated with those found in the literature (Dayani et al., 2011; Câmara et al., 2016; Lestingi et al., 2016).

Table 2. Serum biochemical panel of lambs fed with different levels of whole cottonseed.

Variables[1]	Whole cottonseed, %					Mean or equation[2]	Probability value[3]			SEM[4]	CV[5]
	0	10	20	30	40		WCS	L	Q		
Glucose	79.4	72.8	71.6	72.6	69.2	$\bar{y} = 73.4$	0.5916	0.1716	0.6306	1.97	17.2
Triglycerides	90.3	96.3	96.4	103.9	128.0	$\bar{y} = 100.6$	0.2401	0.0301	0.3165	5.12	30.1
Cholesterol	38.3^{c}	48.9^{bc}	54.8^{b}	59.2^{ab}	71.5^{a}	(1)	0.0019	0.0001	0.9302	2.83	32.0
Proteins	6.36^{abc}	5.19^{c}	6.09^{bc}	8.28^{a}	7.75^{ab}	(2)	0.0360	0.0171	0.3546	0.35	31.7
Albumin	3.09	3.02	3.13	3.81	2.99	$\bar{\bar{y}} = 3.19$	0.0646	0.3782	0.2422	0.10	18.2
ALT	31.3	28.6	34.4	36.9	25.7	$\bar{y} = 31.4$	0.1231	0.6643	0.0725	1.44	27.1
AST	94.5	89.6	95.1	106.2	98.1	$\bar{y} = 96.0$	0.1825	0.1263	0.9688	2.15	13.2
ALP	51.3^{a}	30.6^{b}	37.2^{b}	54.6^{a}	28.5^{b}	(3)	0.0006	0.1604	0.9906	2.62	37.8
GGT	27.3^{c}	46.3^{c}	65.4^{b}	90.0^{a}	83.0^{ab}	(4)	0.0001	0.0001	0.1275	5.17	52.7

Means followed by different letters in the same row differ ($p \leq 0.05$) by t-test.
[1] Glucose, triglycerides and cholesterol (mg/dL); albumin and total proteins (g/dL); ALT, AST, ALP, and GGT (U/L). ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP= alkaline phosphatase; GGT= gama glutamyl transpeptidase. Reference values for lamb: glucose (50-80 mg/dL); triglycerides (undefined); cholesterol (52-76 mg/dL); total proteins (6.0-7.9 g/dL); albumin (2.4-3.0 g/dL); ALT (30 UI/L); AST (307 UI/L); ALP (387 UI/L); GGT (20-52 UI/L) (Feldman et al., 2000).
[2] Means for variables which didn't differ ($p > 0.05$) or regression equation for the variables that differ ($p \leq 0.05$), according to the the levels of whole cottonseed.
[3] WCS= whole cottonseed levels; L= linear tendency; Q= quadratic tendency.
[4] SEM= standard error mean.
[5] CV (%)= coefficient of variation.
(1) $\hat{y}_{Cholesterol} = 39.24 + 0.77WCS$ $(r^2 = 0.97)$; (2) $\hat{y}_{Total\ proteins} = 5.63 + 0.05WCS$ $(r^2 = 0.49)$; (3) $\hat{y}_{ALP} = 44.75 - 0.21WCS$ $(r^2 = 0.081)$; (4) $\hat{y}_{GGT} = 30.17 + 1.62WCS$ $(r^2 = 0.92)$.
Source: Authors.

Of the enzymes that are indicative of hepatocellular extravasation, the values for aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were in agreement with the reference values (Feldman et al., 2000). The alanine aminotransferase (ALT) content was slightly higher (31.37 IU/L) than the reference value (30 IU/L). The activities of the enzyme gamma-glutamyl transferase (GGT) increased ($p \leq 0.05$) with the levels of WCS. From the inclusion of 13.5% WCS ($\hat{y}_{GGT} = 30.17 + 1.62WCS$; $r^2 = 0.92$) in the diet, the GGT results extrapolated the reference values above normal, indicating possible acute liver injury (González et al., 2000). Câmara et al. (2016) evaluated the inclusion of 40% cottonseed cake (3.28 mg.g^{-1} of free gossypol and 0.11 mg.g^{-1} bound gossypol) in the diet of female Santa Inês sheep confined for 63 days. They reported values 29.7 (± 8.8); 144 (± 40.8) and 48.3 (± 5.82) IU/L, for ALP, AST and GGT, respectively; no signs of toxicity caused by gossypol were observed in the studied period.

In the present study, the biochemical constituents evaluated in the serum of the lambs indicated the condition of the nutritional status and liver function of the animals (Feldman et al., 2000). It was inferred that the levels of whole cottonseed that were tested did not affect the nutritional condition of the animals; however, such ingestion promoted important alterations in the levels of enzymes, which were indicative of hepatic function, suggesting that the addition of this ingredient in the diet can cause damage to the liver cells and may cause harm to the health of the animals when fed on whole cottonseed for long periods.

3.2 Skeletal muscle fibres

The results of the effect of time (reference × WCS) and effect of diet (0, 10, 20, 30, 40% WCS) on all the studied variables are shown in Table 3. The variables that differed ($p \leq 0.05$) according to time are presented in Figures 2 and 3; the variable that differed ($p \leq 0.05$) according to the levels of WCS is presented in Figure 3.

Table 3. Characteristics of muscle fibres of *Longissimus thoracis* from lambs fed with different levels of whole cottonseed.

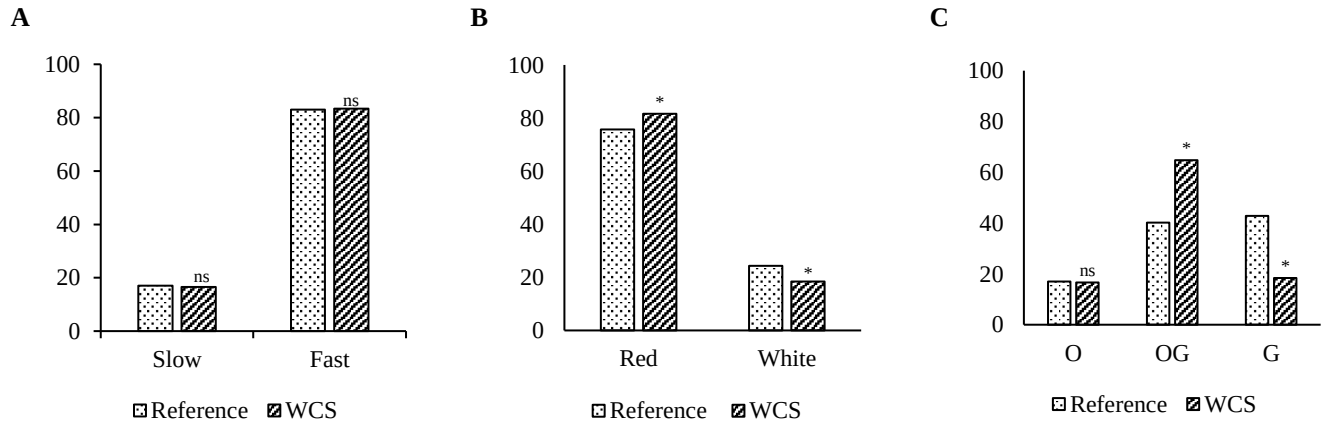
Variables	Reference	Whole cottonseed, %					Mean or Equation[1]	Probalistic value[2]				CV[3]
		0	10	20	30	40		Ref × WCS	WCS	L	Q	
Twitch (%)												
Slow	17.0	16.9	15.5	15.9	17.0	17.6	$\bar{y} = 16.6$	0.8306	0.8142	0.4836	0.3866	24.8
Fast	83.0	83.2	84.5	84.1	83.0	82.4	$\bar{y} = 83.4$	0.8325	0.8240	0.4904	0.3964	4.94
Colour (%)												
Red	75.7	82.2*	79.3	82.5*	84.1*	80.0	$\bar{y} = 81.6$	0.0009	0.2368	0.8237	0.5875	4.78
White	24.4	17.8*	20.7	17.5*	15.9*	20.0	$\bar{y} = 18.4$	0.0008	0.2354	08150	0.5744	20.8
Metabolism (%)												
Oxidative	17.0	16.9	15.5	15.9	17.1	17.6	$\bar{y} = 16.6$	0.8306	0.8142	0.4836	0.3866	24.8
Oxidative glycolytic	58.6	65.3	63.8	66.6	65.6	62.4	$\bar{y} = 64.8$	0.0346	0.6386	0.5368	0.3528	9.58
Glycolytic	24.4	17.8*	20.7	17.5*	15.9*	20.0	(1)	0.0008	0.0429	0.9048	0.3436	18.6
Diameter (µm)												
Type I	15.2	19.3*	18.7*	19.5*	19.8*	19.5*	$\bar{y} = 19.3$	0.0001	0.6915	0.3605	0.8737	10.7
Type IIA	15.6	20.2*	18.7*	19.3*	19.6*	18.8*	$\bar{y} = 19.3$	0.0003	0.5226	0.3952	0.7002	12.1
Type IIB	16.0	20.6*	19.4*	20.0*	20.4*	18.9*	$\bar{y} = 19.9$	0.0001	0.1132	0.3030	0.8104	11.3
Type IIC	14.4	19.6*	18.4*	18.9*	19.8*	18.1*	$\bar{y} = 19.0$	0.0001	0.3143	0.2362	0.7732	11.1
Area (µm²)												
Type I	267	457*	434*	467*	478*	451*	$\bar{y} = 457$	0.0001	0.8087	0.6469	0.7138	21.4
Type IIA	284	486*	423*	446*	450*	420*	$\bar{y} = 445$	0.0008	0.6044	0.3079	0.7126	24.3
Type IIB	303	516*	456*	475*	487*	430	$\bar{y} = 473$	0.0004	0.1598	0.3773	0.7429	23.4
Type IIC	252	458*	403*	431*	466*	391*	$\bar{y} = 431$	0.0001	0.4082	0.1709	0.9843	21.6
Frequency (%)												
Type I	17.5	16.3	15.1	14.3	16.6	16.7	$\bar{y} = 15.8$	0.3248	0.5765	0.5774	0.2207	22.2
Type IIA	39.9	49.1	48.2	49.3	52.4*	42.5	$\bar{y} = 48.2$	0.0148	0.1005	0.2452	0.0895	16.3
Type IIB	22.5	17.1*	19.4	16.8*	16.4*	19.8	$\bar{y} = 17.9$	0.0077	0.0651	0.0438	0.1111	20.9
Type IIC	20.0	17.5	17.2	18.2	17.1	21.0	$\bar{y} = 18.2$	0.2295	0.1432	0.5023	0.3322	17.8
Relative area (%)												
Type I	17.0	16.9	15.5	15.9	17.1	17.6	$\bar{y} = 16.6$	0.8306	0.8142	0.4836	0.3866	24.8
Type IIA	40.1	48.5	47.5	45.0	48.5	41.7	$\bar{y} = 46.3$	0.0888	0.2990	0.1299	0.5650	16.9
Type IIB	24.4	17.8*	20.7	17.5*	15.9*	20.0	$\bar{y} = 18.4$	0.0008	0.0609	0.0194	0.1026	21.7
Type IIC	18.5	16.8[b]	16.3[b]	17.2[b]	17.1[b]	20.7[a]	(2)	0.5648	0.0429	0.9048	0.3436	19.8
Fibre area ratio (µm²)												
IIA/I	1.05	0.90	0.99	0.98	0.95	0.95	$\bar{y} = 0.95$	0.3078	0.8642	0.7909	0.6659	19.5
IIB/I	1.12	0.97	1.05	1.03	1.03	0.97	$\bar{y} = 1.01$	0.1454	0.8995	0.7426	0.4300	15.4
IIC/I	0.95	0.94	0.93	0.95	0.97	0.93	$\bar{y} = 0.95$	0.8863	0.6608	0.8978	0.1547	9.29
Difference in fibre area (µm²)												
IIA-I	16.9	-56.5	-10.7	-20.7	-27.6	-31.7	$\bar{y} = -28.8$	0.2733	0.8735	0.7353	0.4013	-
IIB-I	35.5	-21.2	22.5	7.9	9.3	-21.8	$\bar{y} = -0.19$	0.2994	0.6300	0.8759	0.1560	-
IIC-I	-15.0	-16.4	-30.3	-25.5	-12.3	-28.2	$\bar{y} = -22.6$	0.6659	0.8233	0.9086	0.9303	-

Means in the same line followed by * differ ($p \leq 0.05$) from the Reference by Dunnett's test. Means in the same line followed by distinct letters differ ($p \leq 0.05$) by t-test, depending on the levels of whole cottonseed.
[1] Means for variables didn't differ ($p > 0.05$) by t-test, or regression equation for the variables that differ ($p \leq 0.05$) by t test, according to levels of whole cottonseed.
[2] Modulation= Reference × WCS (0, 10, 20, 30, 40% whole cottonseed); WCS= whole cottonseed levels; L= linear tendency; Q= quadratic tendency.
[3] CV (%)= coefficient of variation.
(1) $\hat{y}_{glycolytic} = 18.48 - 0.002WCS$ ($r^2 = 0.99$), (2) $\hat{y}_{Type\ IIC} = 15.90 + 0.08WCS$ ($r^2 = 0.60$).
Source: Authors.

3.2.1 Modulation of skeletal muscle fibres

The modulation of skeletal muscle fibres consists of the transition from one type of fibre to another in the postnatal period. The time (reference × WCS) of confinement did not affect ($p > 0.05$) the contraction velocity (Figure 1, A); however, it promoted modulation ($p \leq 0.05$) in colour (Figure 1, B), metabolism (Figure 1, C) and in the morphometric characteristics of the skeletal muscle fibres (Figure 2).

Figure 1. Twitch (A), colour (B) and metabolism (C) of *Longissimus thoracis* from lambs fed on different levels of whole cottonseed, as a function of time (reference × WCS).



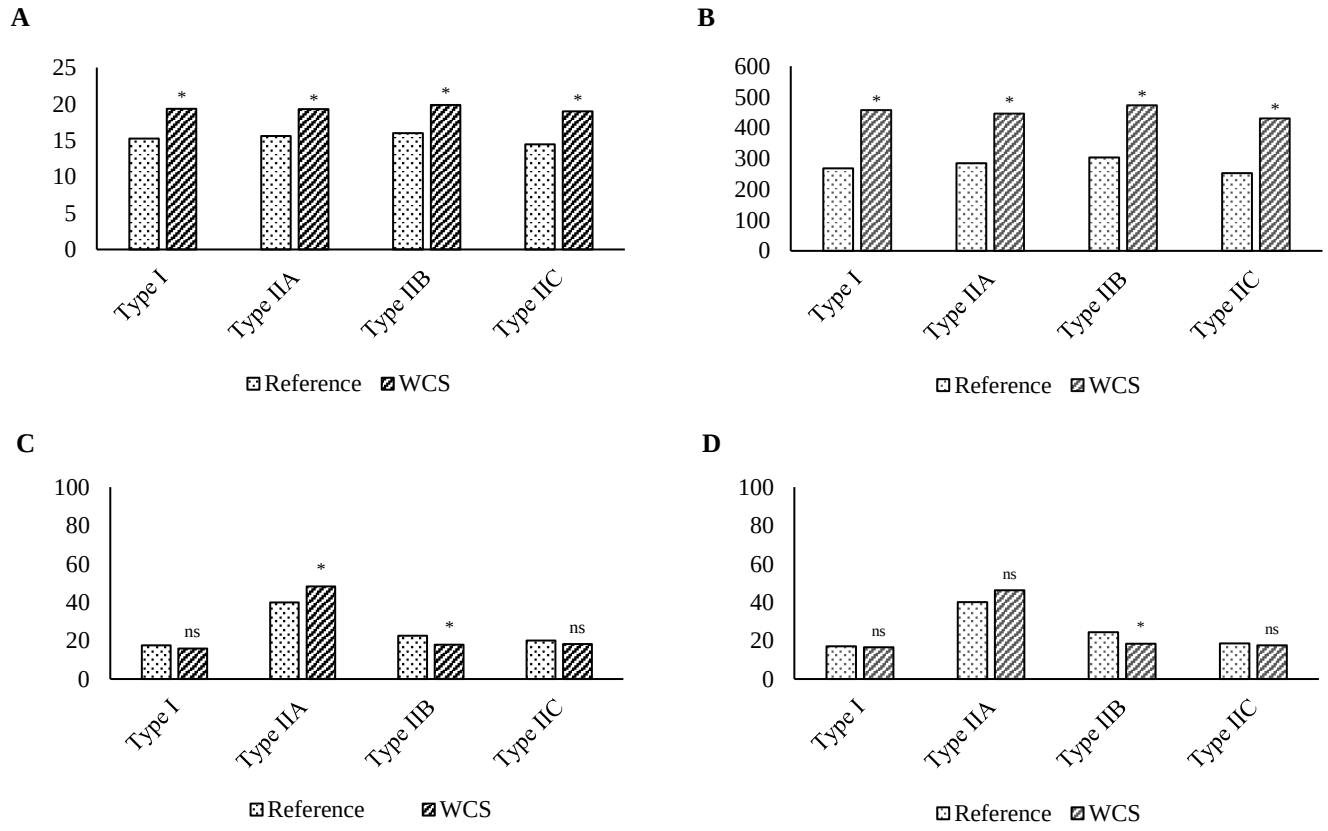
O= oxidative, OG= oxidative glycolytic, G= glycolytic. Data expressed as % of relative area. WCS followed by * differs ($p \leq 0.05$) from Reference by t-test. ns= not significant. Source: Authors.

The time of confinement (reference × WCS) influenced ($p \leq 0.05$) the colour of the muscles, with an increased expression of red fibres and a reduction of white fibres. Glycolytic metabolism generally increases as animals get older due to increases in the diameter of fibres, which reduces oxygen diffusion, thus reducing oxidative metabolism (Picard et al., 2002; Peinado et al., 2004). However, in the present study the feeding time (reference × WCS) promoted an inverse relationship between metabolism and age; there was a reduction ($p \leq 0.05$) of the glycolytic fibres (Type IIB) and an increase ($p = 0.0888$) in the oxidative-glycolytic fibres (Type IIA + Type IIC) (Figure 1, C). This transition was due to the nature of the nutrients supplied to the lambs in confinement because the metabolism of fibres is influenced by the nutritional contribution of the diet. Glycolytic fibres use glycogen reserves (glucose and glycogen), while oxidative fibres use lipid reserves (triglycerides, free and volatile fatty acids, ketone bodies) (Picard et al., 2002).

The metabolic properties of the muscle fibres influence the quality of the meat, its processing, and the quality of its derivatives. In meat processing, slow, oxidative muscles are associated with redder meat; to the highly shortened sarcomere and to the lower intensity of proteolysis, reducing the final meat tenderness (Santello et al., 2010). Thus, muscles with a predominance of oxidative metabolism could benefit more from electrical stimulation (Ithurralde et al., 2015). On the other hand, fast, glycolytic muscles are associated with lower final pH levels in meat and reduced water retention capacity, which can result in lower emulsification capacity (Ithurralde et al., 2015).

As expected, compared to reference the confined animals presented larger ($p \leq 0.05$) diameters (Figure 2, A) and areas (Figure 2, B) in all the types of fibres in *Longissimus thoracis*, with an average increase of 27 and 63%, respectively. Increased fibre size (hypertrophy) in postnatal animal development is associated with factors such as the age at slaughter (Peinado et al., 2004), the type of muscle and its exercise rate (Ithurralde et al., 2015), the method of termination (Santello et al., 2010) and the nutritional quality of the diet (Gallo et al., 2009). Hypertrophy indicates a greater deposition of muscle protein, proving the nutritional efficiency of a diet, and results in higher carcass yield (Chriki et al., 2013).

Figure 2. Diameter, μm (A); area, μm^2 (B); frequency, % (C); and relative area, % (D) of *Longissimus thoracis* from lambs fed on different levels of whole cottonseed, as a function of time (reference × WCS).



WCS followed by * differs ($p \leq 0.05$) from Reference by t-test. ns= not significant. Source: Authors.

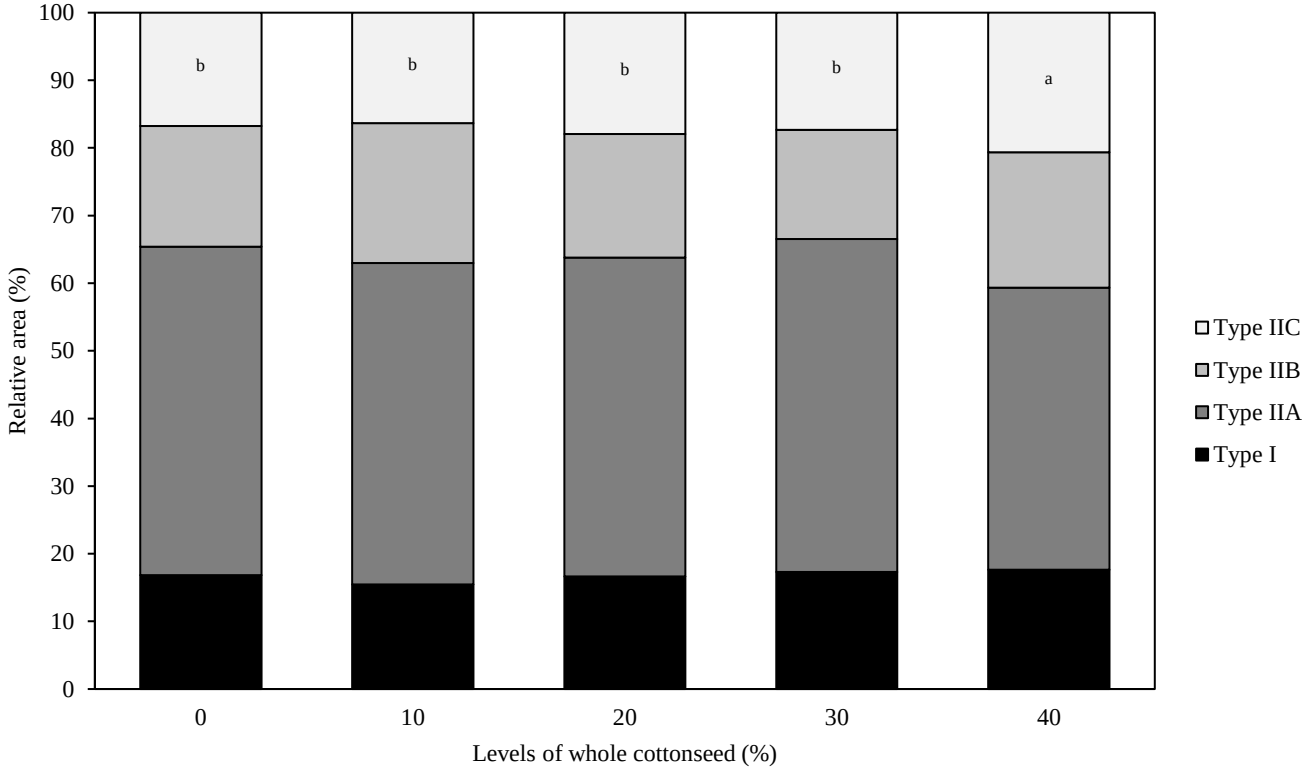
Time altered ($p \leq 0.05$) the proportion of fibre types, which was evidenced by an increase in the frequency of Type IIA fibres and a reduction in Type IIB fibres (Figure 2, C), comparing the Reference to the WCS. The proportion of the area relative to the muscle was altered ($p \leq 0.05$), but only for Type IIB fibres (Figure 2, D). The proportion of fibres in muscles is constantly altered in the postnatal development of animals and may exhibit different behaviours depending on the period that is analysed. The evaluation of *Longissimus thoracis* in postnatal development (1 to 90 days and adults) of adult Segureña lambs showed that modulation in the proportion of fibres varied according to time; comparing the period of one to 30 postnatal days there was a reduction in Type IIA fibres and an increase in Type IIB fibres (Peinado et al., 2004). At the stage of 30 days, an inverse relationship was observed in the adult animals, i.e. an increase in Type IIA fibres and decreased numbers of Type IIB fibres.

3.2.2 Effect of diet on the performance of skeletal muscle fibres

The increase of whole cottonseed in the lambs' diet reduced ($p \leq 0.05$) glycolytic metabolism ($\hat{y}_{glycolytic} = 18.48 - 0.002WCS$; $r^2 = 0.99$) and not altered ($p > 0.05$) the contraction velocity and colour of the fibres (Table 3). The *Longissimus thoracis* of the lambs fed on WCS was characterised as fast-contracting muscle (83.43% rapid vs. 16.59% slow), red (81.63% red vs. 18.38% white) and oxidative-glycolytic metabolism (16.59% oxidative, 64.76% oxidative-glycolytic, 18.65% glycolytic). These results agreed with those previously reported for *Longissimus thoracis* from sheep, which were classified as rapid and red (Briand et al., 1981); however, they differed in terms of metabolism, which was defined as glycolytic by Ithurralde et al. (2015). This difference can be attributed to factors such as the degree of physiological maturity of the animals and the portion of the muscle that was sampled, as well as the termination system.

In terms of the morphometric characteristics (diameter, area, frequency and relative area) of the *Longissimus thoracis* muscle fibres (Table 3), only the relative area of the Type IIC fibres was altered ($p \leq 0.05$) by the ingestion of WCS ($\hat{y}_{IIC} = 15.90 + 0.08WCS;\ r^2 = 0.60$) (Figure 3), was due to the diet being rich in lipid substrate.

Figure 3. Relative area (%) of *Longissimus thoracis* from lambs fed on different levels of whole cottonseed, as a function of level of whole cottonseed.



Means of the same fibre type followed by a different letter differ ($p \leq 0.05$) by t-test according to the level of whole cottonseed. $\hat{y}_{Type\ IIC} = 15.90 + 0.08WCS, r^2 = 0.60$. Source: Authors.

The literature reports differences in the size of different fibre types; this depends on factors such as diet, type of muscle, and the age at slaughter. The *Longissimus thoracis* of adult lambs showed a difference in diameter between fibre types, with Type IIA fibres (20.54 μm) being smaller than Type I (26.33 μm) and Type IIB (24.27 μm) fibres (Peinado et al., 2004). The semitendinosus muscle of ½ Dorper-Santa Inês lambs finished in different feeding systems (confinement and grazing plus oil supplementation) had the largest diameters for oxidative-glycolytic fibres (43.71 μm), followed by glycolytic fibres (35.96 μm) and oxidative fibres (20.34 μm) (Santello et al., 2009). Lambs fed on sunflower grain (9.10%) presented similar sized diameters for red fibres (35.38 μm), intermediate fibres (36.02 μm) and white fibres (35.31 μm) in relation to the semitendinosus muscle. However, for the *Longissimus lumborum* muscle the diameters of the fibre types presented some differentiation, with values of 28.71, 29.81 and 32.22 μm for red, white and intermediate fibres, respectively (Santello et al., 2010). Contrary to the aforementioned results, in the present study the diameter and area of the fibres did not vary depending on the fibre types (I, IIA, IIB and IIC), with mean values of 19.4 μm and 452 μm^2, respectively (Table 3). The lack of differentiation between fibre types in terms of diameter, and the lower values that were observed compared to those reported in the literature, may indicate incomplete physiological maturity of the animals, i.e. the muscle growth of the lambs was still very active (Macedo et al., 2000).

The frequency of the types of fibre did not vary ($p > 0.05$) according to the levels of WCS in the diet; the *Longissimus thoracis* muscle contained a higher proportion of intermediate fibres (Type IIA + Type IIC = 66.4%), followed by glycolytic

fibres (Type IIB = 17.9%) and oxidative fibres (Type I = 15.8%). The proportion of the relative area of the fibre types followed the same pattern as the frequency, with mean values of 46.3, 36.0 and 16.6% for intermediate, glycolytic and oxidative fibres, respectively. These results were consistent with earlier studies that demonstrated that short-term dietary systems did not influence the proportion of fibres in ovine muscles (Gallo et al., 2009; Santello et al., 2009; Santello et al., 2010).

3.3 Relationship between muscle fibre and meat quality

Meat consists of innumerable tissues, predominantly muscular tissues, which are composed of muscular fibres. Therefore, changes in the types of muscle fibres have a direct impact on the quality of the meat. Quality has been defined as product performance that results in consumer satisfaction; the main intrinsic attributes associated with meat quality are colour, flavour, texture, conservation (shelf life), chemical composition, reliability and convenience (Chriki et al., 2013; Joo et al., 2013; Ponnampalam et al., 2016).

The characteristics of the muscle fibres of *Longissimus thoracis* were correlated with the qualitative characteristics of the lamb meat. The correlations showed a low or medium degree of association (low < 0.40 ≥ mean ≤ 0.70 > high). Only the significant variables and correlation coefficients ($p \leq 0.05$), and those with practical significance for this study, are presented (Table 4).

The colour of meat is the first quality factor evaluated by consumers (Ponnampalam et al., 2016). As expected, the characteristics of red fibres were positively correlated with deoxymyoglobin (Type I, $r = 0.51^{*}$) and red intensity (Type IIA, $r = 0.47^{*}$), while the white fibres (Type IIB) was negatively correlated with red intensity and colour saturation. Myoglobin adopts a deoximioglobin structure when meat is stored in an anaerobic environment, and it promotes a cherry red colour for meat (Ponnampalam et al., 2016). Thus, these results were in agreement with the colour characteristics of the fibres, since Type I and Type IIA fibres have a higher myoglobin content than Type IIB and Type IIC fibres (Choe & Kim, 2014). Although Type I fibres have a higher lipid content, the diameter and area of these fibers was inversely correlated with the intensity of yellow.

The water retention capacity was positively correlated (r = 0.37*) with the diameter of the oxidative fibres (Type I). Similarly, Ryu & Kim (2005) associated an increase in water retention capacity with the number of Type I and Type IIA fibres, due to the inverse correlation of these fibres with drip losses in *Longissimus dorsi* of pork. Water retention capacity influences the quality of meat, both when it is fresh and also after cooking, because it is closely related to the colour of fresh meat and the softness of cooked meat (Ryu & Kim, 2005; Ponnampalam et al., 2016).

In relation to texture, the Type IIA fibres (diameter and area) were negatively correlated ($r = -0.37^{*}$) with the Warner-Bratzler shear (WBS) values, in other words, the larger the diameter and area of the Type IIA fibres, the lower the hardness (i.e. softer). Likewise, the Type IIA fibres presented a negative correlation ($r = -0.38^{*}$) with cohesiveness, demonstrating that an increase in the frequency of this type of fibre decreases the cohesiveness (softer). Such behaviour indicates that, considering the same area of muscle tissue, the larger the diameter of the fibres, the fewer fibres that were present in this interval. Consequently, less connective tissue to permeate the fibres (endomysium) or fibre bundle (perimysium) was required to be ruptured for the shear force and cohesiveness tests, which directly influenced the softness of the meat. Thus, it can be inferred that Type IIA fibres were associated with desirable characteristics regarding the texture of the meat.

Table 4. Partial Spearman's correlation between the characteristics of muscle fibres of *Longissimus thoracis* versus the quality of meat from lambs fed with different levels of whole cottonseed.

Variables[1]	Type I				Type IIA				Type IIB				Type IIC			
	d	Area	F	RA	d	Area	F	RA	d	Area	F	RA	d	Area	F	RA
*a**	-0.55***	-0.49***						0.47*	-0.17*	-0.12*			-0.44*	-0.41**		
*b**	-0.51**	-0.44*														
C	-0.55***	-0.49**						0.48*	-0.19*	-0.13*			-0.41*	-0.37**		
DMb	0.57*	0.51*														
pH 0h			-0.34*				0.30*	0.31*						-0.31*		
A_w							0.41*								-0.47*	
WHC	0.37*															
WBS					-0.37*	-0.37*										
Tn			-0.38*	-0.41*												
coe							-0.38*									
OF				0.37*				-0.39*								
MF			0.37*													
SF	-0.39*	-0.40*												-0.38*		
LF			0.42*	0.39*			-0.41*	-0.41*							0.37*	
FF	-0.51**	-0.45											-0.40*			
Lipids												0.40*				
VLCFA							0.38*				-0.41*	-0.38*				
OCFA															-0.38*	
SFA			0.42*	0.43*				-0.44*								
MUFA										-0.37*						
UFA			-0.42*	-0.43*			0.45*	0.44*								
MS			-0.37*				0.38*									
*n*3			-0.48**				0.58**	0.50**			-0.37*					
*n*6: *n*3			0.43*				-0.48**	-0.40*								
IT			0.43*	0.43*			-0.48**	-0.48**								
NR			0.39*													

[1] d= diameter; F= frequency; RA= relative area; *a**= redness; *b**= yellowness; C= chroma; DMb= desoxymyoglobin; A_w= water activity; WHC= water-holding capacity; WBS= Warner-Bratzler shear; Tn= tenderness; coe= cohesiveness; OF= off flavour; MF= metallic flavour; SF= sweet flavour; LF= liver flavour; FF= fat flavour; VLCFA= very long-chain fatty acids; OCFA= odd-chain fatty acids; SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; UFA= unsaturated fatty acids; MS= MUFA/SFA ratio; *n*3= omega-3; *n*6: *n*3= omega-6/omega-3 ratio; IT= index of thrombogenicity; NR= nutritional ratio. *significant ($p \leq 0.05$); **significant ($p \leq 0.01$); ***significant ($p \leq 0.001$). Source: Authors.

Meat from sheep can present undesirable sensorial characteristics, such as a flavour and aroma that is more intense than usual. The latter has been associated with factors such as feeding, physiological condition, castration and pre-slaughter stress (Monte et al., 2012). In the present study, the correlations between sensory attributes and fibre characteristics showed that undesirable aspects (off-flavour, metallic taste and liver taste) were associated with the presence of Type I fibres. An increase in Type IIA fibres was associated with a reduction in off-flavour ($r = -0.39^{*}$) and liver taste ($r = -0.41^{*}$).

The consensus in the literature is that lipid content is high in Type I fibres, high or intermediate in Type IIA fibres, and low in Type IIB fibres (Picard, 2012; Chriki et al., 2012; Choe & Kim, 2014). However, in the present study, the lipid content in *Longissimus thoracis* only presented a positive correlation ($r = 0.40^{*}$) with the relative area of Type IIB fibres. In relation to the fatty acid profile, the Type I fibres (slow, oxidative) were associated with undesirable aspects (increased saturated fatty acids, reduced unsaturated fatty acids, a lower monounsaturated:saturated ratio, lower *n*3, higher *n*6:*n*3 relationship, and higher thrombogenicity index); while the Type IIA (intermediate) fibres were associated with desirable characteristics of the fatty acid profile (increased unsaturated fatty acids and *n*3, and reduced saturated fatty acids, *n*6:*n*3 ratio, and thrombogenicity index).

4. Conclusion

The addition of whole cottonseed to the diet of lambs promoted changes in the activities of liver enzyme function, suggesting that the addition of this ingredient may have caused damage to liver cells.

The time of confinement positively influenced the modulation of the skeletal muscle fibres, promoting an increase in the diameter and area of all the fibres; this factor was more pronounced than the effect of the level of whole cottonseed in the diet.

The quality of the meat was influenced by the characteristics of the different fibres types of *Longissimus thoracis*; the intermediate fibres, which were present in greater quantity in the muscle, were positively correlated with the desirable quality characteristics of the meat (texture, sensorial, fatty acid profile).

Further research is required in this field and should be conducted on specific factors such as modulation studies in different muscles, longer times and with heavier animals, as well as using different ingredients in the diet aiming at improving meat quality.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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