Natural antioxidants incorporated into *Longissimus dorsi* muscles of pasture or grain fed steers and their relation to gene expression

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Abstract— **The aim of this work was to study the prooxidant/antioxidant behavior in *longissimus dorsi* (LD) muscle and the concomitant gene expression related to the incorporation of antioxidants. For this reason, two groups of crossbreed steers were fed either pasture diet (P): reared on winter pastures and finished on alfalfa or corn silage diet (C): 68% corn silage, 15% sunflower flour, 5% hay, 2% vit/mineral. Significant differences ($P<0.05$) for α-tocopherol and β-carotene were 2.24±0.44 vs. 1.66±0.49; 0.16±0.04 vs. 0.06±0.02 respectively for P vs. C samples. No differences were found for retinol, lutein and γ-tocopherol. Additionally, the effect of diet on antioxidant capacity (AOC) of hydrophilic and lipophilic antioxidants was evaluated using the FRAP-ferric reducing ability. AOC showed similar values for both groups. In addition, AOC values of the hydrophilic antioxidants were significantly higher than lipophilic values. TBARs values were similar for P and C groups in fresh samples. However, after the induction of oxidation with $FeSO_4$ and ascorbic acid, P samples showed a higher resistance to oxidation compared with C samples. Gene expression was determined in mRNA isolated from LD tissue by RT-PCR. Selected genes corresponded to: AOX2P: Non-selenium glutathione phospholipid-peroxidase; αTAP: α-tocopherol associated protein; α-TTP: α-tocopherol transfer protein and CRBP1: retinol binding protein. Transcription assays showed that signals for α-TAP and α-TTP were less significant for P compared to C samples. Neither AOX2P nor CRBP1 showed differential expression between diets. Pasture feeding enhanced the resistance to oxidation and could modulate gene expression related to tocopherol incorporation into LD muscle.**

Keywords— **tocopherol, gene expression, pasture feeding.**

I. INTRODUCTION

Natural antioxidants are incorporated into meat through dietary delivery. Many studies indicate that pasture feeding and rich-antioxidant supplements are the most important vehicles to enhance the antioxidant activity in muscle tissues [1]; [2]; [3]; [4]. Most of them may enhance or preserve the vitamin E content and its capacity to counteract the prooxidant effect of free radicals. Other authors showed the effectiveness of polyphenol rich extracts combined with vitamin E to enhance the oxidative stability of LT and ST muscles [5]. Polyphenols, vitamin C and E and carotenoids are natural constituents of fresh pasture and this way they may serve to preserve the tissues from lipid and protein oxidation [6]; [7]; [8]; [9]. Once into the tissues, the antioxidant network controls oxidation. The adsorption of fat-soluble vitamins A, E and carotenoids occur within the small intestine of ruminants. They are incorporated into chylomicrons and transported into the liver. In the case of retinol, the retinol-binding protein (CRBP1) may bind retinol and deliver it from the liver to the tissues. This pathway can be modulated in plasma by dietary vitamin A in calve [10]. In the case of vitamin E, the liver tocopherol-transfer protein (α-TTP) binds the alpha isomer in a selective way and this is the reason of a α-tocopherol to be the most abundant form of the vitamin E in mammalian tissues. The expression of a cytosolic protein, α-TAP (SEC-14 like protein), could be related to the intracellular trafficking of vitamin E [11]; [12]. Therefore the incorporation of fat-soluble antioxidants into the muscle can be regulated by

specific proteins that incorporate these molecules into the cells.

II. MATERIALS AND METHODS

A. Animals and diets

Twelve crossbreed steers were grown at INTA experimental station located at Anguil, La Pampa, Argentina. As growth rate differed for corn silage or pasture- fed animals, the latter continued on trial until slaughter weight was reached (approximately 480 kg weight). Six animals per group were fed either Pasture diet (P): reared on winter pastures and finished on alfalfa or corn silage diet (C): 68% corn silage, 15% sunflower flour, 5% hay, 2% vit/mineral. LD samples were kept at -80ºC until analysis. All procedures used complied with national regulations concerning experimentation on farm animals.

B. Oxidative stability, antioxidant vitamins and FRAP assay

Oxidation was determined by TBARS in fresh homogenates of LD muscle or after induction with $FeSO_4$ and ascorbic acid for 30 min at 37 ºC. Total antioxidant capacity (AOC) was evaluated using the FRAP-ferric reducing ability assay either in hydrophilic and lipophillic homogenates fractions. Hydrophyllic antioxidants were extracted from homogenates (1:2 w/vol) with phosphate buffer 0.05M, pH 7.8. Lipophillic fractions were obtained by extraction of homogenates with acetic-ethanol solution (1:3 vol/vol) [13]. Fat-soluble vitamins were extracted with n-hexane after saponification and determined by HPLC-UV/vis and fluorescence detection as described previously [14]. All chemicals were HPLC grade (J.T. Baker, Argentina). Ascorbic acid, vitamin standards, thiobarbituric acid, ferric chloride, ferrous sulfate and TPTZ were purchased from Sigma-Aldrich, Argentina.

C. Gene transcription assays.

Gene expression was determined in mRNA isolated from LD samples by semiquantitative RT-PCR. Selected genes corresponded to: AOX2P (AOP2): Bos taurus anti-oxidant protein 2 (non-selenium glutathione peroxidase); α-TAP: α-tocopherol associated protein; α-TTP: α-tocopherol transfer protein and CRBP1: retinol binding protein. The primers were designed at the University of California, Davis and purchased from IDT (CA, USA). As housekeeping genes 18s RNA was used. NCBI nucleotide bovine database and the Primer 3 program were used to design specific primers (table 1). Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. DNAse I treatment was applied to remove traces of contaminating DNA. RNA obtained was reverse transcribed to cDNA using MuLV Reverse Transcriptase (Promega). cDNA products were amplified with Taq DNA Polymerase (Invitrogen), in presence of specific primers (Table 1). Images of RT-PCR agarose gels which were stained with ethidium bromide were acquired, and the bands were quantified using the software Image J. Semiquantitative expression was determined using the imagine software on gels pictures and each value was standardized to the corresponding 18s signal. It was also indicative of the reaction efficiency.

Table 1 Primers used for mRNA transcription assays

Gene/ Accesion number		Primer (5'-3')
AOX2P	F	AGGCATGAGCAACATGACTG
(NM_174643)	R	AATATCTCCCTCCCCCACTG
α-TAP	F	GCATCTATGTCCTGCGGTTT
(AF_432353)	R	CCCCAACTGTTGCATCTTCT
α-TTP	F	CACACTGGGACCCAAAAGTT
(XM_587081)	R	TGAAAGGCATGACGAAACTG
CRBP1	F	AGGACGGGGACCAGTTCTAC
(NM_001025343 /AF502256)	R	CCCCTCCAGAAGAGTTTGTG
18S	F	AAACGGCTACCACATCCAA
(AF176811)	R	CGCTCCCAAGATCCAACTAC

D. Statistical analysis.

All data were analyzed using one way ANOVA and t statistic for differences between treatments or two-way ANOVA when stated with the SAS, Inc, USA.

III. RESULTS AND DISCUSSION

Pasture feeding induced higher incorporation of α-tocopherol, β-carotene into the LD muscle as

compared to concentrate diet. The other antioxidants, retinol, lutein and γ-tocopherol showed no significant differences attributable to the different diets (figure 1). The levels of the fat-soluble antioxidants were lower than the levels reported previously [7]; [8]. This could be the reason why TBARS levels in fresh meat were not significantly different among diets (TBARS basal; figure 1). However, natural antioxidants contributed to counteract the oxidation when ferrous iron and oxygen peroxide were used to induce a Fenton reaction within the homogenates (TBARS induced; figure 1).

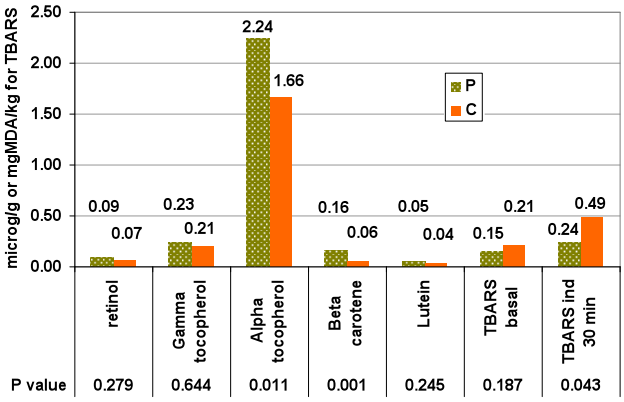

Fig.1: antioxidants and oxidation indicators in pasture (P) or corn silage (C) meat samples.

The total antioxidant capacity (AOC) was determined using the FRAP reaction in lipophilic and hydrophilic fractions of beef samples. AOC showed similar values for both groups (table 2). In addition, AOC values of the hydrophilic antioxidants were significantly higher than lipophilic values. This result differed from previous results obtained with *Psoas major* samples in a similar experiment [14], where FRAP values were higher in pasture meat. Probably a reaction time of 4 min. for AOC determination resulted scarce for the differentiation of treatments, as stated by other authors [13].Gene expression assays were performed from the same beef samples which were analyzed for their biochemical properties. RT-PCRs were performed for each primer set, including the 18s RNA as housekeeping gene to control the endogenous expression.

Table 2 Total antioxidant capacity (AOC) using FRAP reaction in lipophillic and hydrophilic meat homogenates.

Diet	AOC	mM/g fresh tissue
Pasture	hydrophilic	13.22[a]
	lipophilic	8.33[b]
Corn Silage	hydrophilic	13.44[a]
	lipophilic	10.36[b]

[a] Different letters within column indicate significant differences (P< 0.05)

As shown in Fig.2 and Table 3, retinol-binding protein (CRBP1) expression was similar for both treatments. The result was expected as there were not significant differences in the quantity of this vitamin determined in the samples. A similar result was observed for the antioxidant protein (AOX) similar to glutathione peroxidase non-selenium dependent. mRNA signals were similar for both treatments. Surprisingly, the expression of α-Tocopherol transfer protein (α-TTP) and α-tocopherol associated protein (α-TAP or SEC-14 like protein) were lower in pasture samples when compared with corn silage samples.

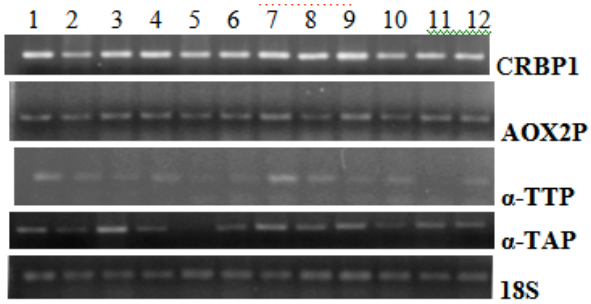

Fig. 2: RT-PCR products for CRBP1 (cellular retinol binding protein 1), AOX (AOP2, antioxidant protein 2),α-TTP (α-tocopherol transfer protein), α-TAP (α-tocopherol associated protein), and 18S (ribosomal αl subunit 18s, housekeeping gene) on 1,8% agarose gel electrophoresis. Lines 1,3,5,6,8,9,11 pasture meat; Lines 2,4,7,10,12 grain meat.

Transcriptional assays could indicate that under productive conditions, α-tocopherol is transferred into the tissues and that the presence of the alpha isomer within the cells could modulate its own delivery through the expression of specific transporters, depending on the dietary offer of this vitamin. However quantitative Real-time PCR analyzes should

be performed on these genes related to tocopherol incorporation into LD tissue.

Table 3 Semiquantitavive mRNA expression in LD samples related to 18s mRNA used as housekeeping gene.

Relation	CRBP1/18s	TTP/18s	AOX/18s	TAP/18s
Pasture	1.09163	0.48830	1.15702	0.94059
Corn Silage	1.18804	0.95912	1.31154	1.17896
P value	*0.68435*	*0.03480*	*0.18480*	*0.03124*

IV. CONCLUSIONS

These results present for the first time the expression of tocopherol transport genes in beef samples obtained under productive conditions. The overall antioxidant content in LD muscles was higher in pasture than in corn silage fed cattle, and this condition prevented the oxidation induced by a Fenton reaction in beef samples.

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