Indian Journal of Animal Sciences **89**(9): 983–991, September 2019/Article
https://doi.org/10.56093/ijans.v89i9.93779

Antioxidant capacity, lipid oxidation status and expression of specific selenoprotein mRNA in *Longissimus dorsi* muscle of lambs (*Ovies aries*) supplemented with supranutritional selenium

S S NONGKHLAW[1], R U SUGANTHI[2], J GHOSH[3], P K MALIK[4], V B AWACHAT[5], P KRISHNAMOORTHY[6] and D T PAL[7]

ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka 560 030 India

Received: 30 October 2018; Accepted: 15 February 2019

ABSTRACT

Selenium is known to play a key role in maintenance of redox status of tissues, immunity, reproduction, thyroid and muscle functions. The present investigation was carried out to understand the effect of supranutritional dietary Se supplementation on antioxidant capacity, lipid oxidation and expression of specific selenoprotein mRNA in *Longissimus dorsi* muscles of growing lambs. Twenty male lambs of 5–6 months of age were fed basal diet supplemented with 0.5, 1.5 or 4.5 ppm Se-yeast (organic Se) or without Se (control) for 90 days. The antioxidant capacity, lipid oxidation of meat during different days of storage and the mRNA expression of GPX1, GPX2, GPX3, TXNRD1, TXNRD3, DIO1, DIO2, DIO3, SEPP1, SEP15 and SEPW1 were studied in *Longissimus dorsi* muscles of sheep. The results indicated improvement in antioxidant status by supplementation of 1.5 and 4.5 ppm Se, and reduction in meat lipid oxidation status on day 0 without any further reduction after 3 and 7 days of storage in all the Se supplemented lambs. A selective change in expression of GPX2, GPX3, TXNRD1, DIO2, DIO3, SEPP1, SEP15 and SEPW1 mRNA was observed by supranutritional Se while GPX1, TXNRD3, DIO1 and expressions remained unaffected by supplementation. In conclusion, supranutritional Se supplementation in lambs increased antioxidant status, reduced lipid oxidation status with limited effect on oxidative stability of meat during storage and regulated *Longissimus dorsi* muscle selenoprotein mRNA expression differentially depending on the Se feeding levels. Our results thus provided new insights into the regulation of selenoprotein gene expression by supranutritional levels of dietary Se.

Key words: Antioxidant, Lambs, Lipid oxidation, *Longissimus dorsi* muscle, Selenoprotein genes, Supranutritional selenium

Selenium (Se) is a well-known essential trace element for humans and animals. Deficiency of Selenium is associated with white muscle disease, oxidative stress and immuunosuppression (Gandhi *et al*. 2013) while Se toxicity leads to alkali disease and mortality in livestock (Kieliszek and B³azejak 2016).

Sheep are a major source of meat throughout the world. The demand for sheep meat is projected to increase substantially in the near future with the forecasted increase in global meat consumption. Driven by an increase in the income, this demand is mostly expected from developing countries (Montossi *et al*. 2013). One of the major problems that affect meat quality is peroxidation of meat lipids which reduces its nutritional quality and accumulates products that are toxic to humans (Min and Ahn 2005). As a dietary supplement, Se improves antioxidant capacity and oxidative stability of meat in broilers (Oliveira *et al*. 2014) and pigs (Calvo *et al*. 2017). It is not known if similar benefits can be obtained in sheep.

Selenoproteins, characterized by the presence of the 21st amino acid the selenocysteine, are deemed to mediate the physiological functions of Se. Mammals possess at least 24 to 25 selenoproteins genes (Mariotti *et al*. 2012). Amongst these, the most studied selenoprotein families are the glutathione peroxidases (GPXs), thioredoxin reductases (TXNRDs) and iodothyronine deiodinases (DIOs). Newer selenoproteins have been identified, including, selenophosphate synthetase 2, methionine sulfoxide reductase, and selenoproteins I, M, N, V, O, H, T, K, and S. However, the functions of many of them are not deciphered (Juszczuk-Kubiak *et al*. 2016). A wide range of tissues including liver, muscle, thymus and brain express selenoprotein genes but their expression pattern differs between tissues and species (Liu *et al*. 2012). Dietary Se concentration influences selenoprotein mRNA abundance

Present address: [1]PG Scholar (shem.sngewbha69 @gmail.com), [2,3,7]Principal Scientist (R.Umayasuganthi @gmail.com, jghosh.office @gmail.com, dtpal@yahoo.co.in), [4]Senior Scientist (malikndri@gmail.com), [5]Assistant Chief Technical Officer (vaibhav.cari@gmail.com). [6]Senior Scientist (krishvet@gmail.com), ICAR-National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India.

in different tissues of pigs (Liu *et al*. 2012), poultry (Liu *et al*. 2014) and goats (Zhang *et al*. 2013a). However, information on regulation of selenoprotein gene expression in response dietary Se changes is limited in sheep.

The organic form of Se is preferred in livestock industry due to its high effectiveness, less toxicity and environment friendliness as compared to inorganic, sodium selenite. Selenium yeast constitute valuable source of easily assimilable Se and the true digestibility of organic Se in the form of Se-yeast is about 66% whilst that from selenite salt is about 50% in sheep (Weiss and Hogan 2005). Supranutritional supplementation of Se, i.e. feeding above the FDA permitted levels but less than the maximum tolerable level (>0.3 and < 5 mg/kg diet) has created interest among the nutritionists to harvest the benefit of positive potential of Se for health improvement of animals (Hugejiletu *et al*. 2013). Supplementation of supranutritional Se reversed the foot-rot disease induced gene expression changes in innate immunity genes in whole blood neutrophils (Hujejiletu *et al*. 2013) and improved resistance to *H. contortus* gastrointestinal parasitism in sheep (Hooper *et al*. 2014). However, there are no studies on oxidative stability of meat or selenoprotein gene expression of sheep fed supranutritional Se.

Keeping the above background in mind, this feeding experiment was designed to understand the effect of dietary supranutritional supplementation of Se on the antioxidant capacity, lipid oxidation and mRNA expression of specific selenoproteins in *Longissimus dorsi* muscle of lambs so that this practice can be recommended to the farmers for improvement of meat quality.

MATERIALS AND METHODS

Animals: Twenty male lambs of a local breed, aged 5–6 months with average body weight of 11 kg were used for this experiment. All the animals were housed in a well-ventilated shed with asbestos roofing and concrete flooring. Before initiation of experiment, the animals were adapted to the environment and experimental diet for 2 months. Necessary prophylactic measures were taken to protect the animals from common bacterial and other ecto-and endo-parasitic infestations. The experiment was conducted at the experimental livestock unit of the Institute after obtaining necessary approval from the Institutional Animal Ethics Committee.

Diet: Experimental animals were fed basal diet comprising concentrate mixture and finger millet straw in the ratio of 50:50, formulated to meet the nutrient requirements as per the Indian Council of Agricultural Research (ICAR-NIANP 2013) standards except for Se. The composition of concentrate mixture of basal diet fed to animals is given in Table 1. Selenium content of basal diet was analyzed by Inductively Coupled Plasma Mass Spectrometry at Indian Institute of Crop Processing Technology, Ministry of Food Processing and Technology, Tamil Nadu, Government of India. The Se content of concentrate and finger millet straw were 18 and 3 ppb, respectively.

Table 1. Ingredient composition of concentrate fed to experimental animals

Ingredient	Proportion
Maize	38
Soybean meal	15
Ground nut cake	17
Wheat bran	27
Mineral mixture*	2
Salt	1
Total	100

*Dicalcium Phosphate, 500 g/kg; Calcite, 170 g/kg; $MgSO_4$, 130 g/kg; NaCl, 133 g/kg; $MnSO_4$, 17 g/kg; $ZnSO_4$, 33 g/kg; $CuSO_4$, 5 g/kg; $COCl_2$, 0.2 g/kg; $FeSO_4$, 12 g/kg

Experimental design and feeding schedule: The experimental lambs were divided into four groups of five animals each based on their body weight following a completely randomized design. The four groups of lambs were fed with either basal diet (control) or the basal diet supplemented with 0.5 ppm Se (Se-0.5 group), 1.5 ppm Se (Se-1.5 group) or 4.5 ppm Se (Se-4.5 group) for 90 days. The animals were stall fed individually with the weighed amount of respective diets on a daily basis throughout the experimental period. The Se-yeast (SEL-PLEX, Alltech Biotechnology Private Limited, India) used as organic Se source had a guaranteed analysis of 2 g/kg of organically bound Se and it was mixed with concentrate to obtain appropriate amount of Se in the diet and fed to animals. All the doses of Se used in the study were below the maximum tolerable level (5 mg/kg as fed) for small ruminants (NRC 2007). Each animal were given free access to clean and fresh water separately.

Collection of tissue samples: At the end of feeding experiment, the lambs (average body weight 15 kg) were sacrificed. About 0.5 g of fresh LD muscle tissue samples were collected in microfuge tubes containing RNAlater (Invitrogen™) for gene expression studies. The tissue samples in RNAlater were kept at 4°C overnight and then transferred to –80°C after decanting the solution for long term storage. Portion of fresh LD muscles were also collected in 10% formalin for histology studies. At 24 h post-mortem, LD muscles from each lambs were cut and the samples were used for determination of antioxidant capacity and quantification of lipid peroxidation products.

Determination of total antioxidant capacity: About 1 g of muscle samples were minced and homogenized in 10 mL of ice-cold distilled water using Tissue ruptor (Qiagen, Germany) at 10 cycles of 30 sec pulses. The homogenates were centrifuged at 2500 × g for 20 min at 4°C to discard the precipitate. The supernatant was filtered through Whatman filter paper #54. The total antioxidant capacity was measured in the filtrate by ferric reducing antioxidant power (FRAP) assay following the method of Benzie and Strain (1966). The test samples were prepared by adding 100 µL of the muscle homogenate, 300 µL of distilled water and 3.0 mL of FRAP reagent.

Determination of lipid peroxidation products in vacuum packed meat: Three slices of meat weighing about 20 g were taken from last four ribs of individual lamb carcass, vacuum packed individually and stored at 4°C. At 0, 3 and 7 days post packing, lipid oxidation status was measured in each samples by determining malondialdehyde (MDA) levels as per the method of Luciano *et al*. (2011).

Designing of primers: The quantitative PCR primers for the selenoprotein and the endogenous control genes (Table 2) were designed from the sequences of each genes available in NCBI data base (https://www.ncbi.nlm.nih.gov/nuccore), using Primer 3 web tool (http://primer3.ut.ee/) and synthesized from the local service provider (Xcelris Labs Limited, Ahmedabad, India).

Isolation of total RNA and determination of quality and quantity: Total RNA from about 100 mg LD muscle tissues were isolated using TRI reagent (Sigma Aldrich, USA) following the manufacturer's protocol. The isolated RNA was further cleaned up using RNeasy mini kit (Qiagen, Germany) and the contaminating DNA if any was removed by in column digestion using DNase set (Qiagen, Germany). The quantity of total RNA were determined by taking absorbance at 260 and 280 nm in Nano Drop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA). The quality of total RNA was determined by 1% agarose gel electrophoresis by separating in MOPS buffer after denaturing at 70°C for 10 min in loading buffer.

cDNA synthesis: The cDNA was synthesized from total RNA by reverse transcription using Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics, Switzerland) as per the manufacturer's protocol. The synthesized cDNA was stored at –80°C for long term storage or at 4°C for short term storage.

Relative quantification of mRNA expression by SYBR green based qPCR assay: The expression of different selenoprotein genes in each sample was measured relative to the expression of endogenous control (reference) gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The efficiency of the endogenous control and test gene assays were determined in 5 folds serially diluted cDNA template starting from 50 ng to 0.4 ng in duplicate with triplicate NTC and negative RT (10 ng total RNA). The PCR amplification of endogenous control and the test genes in actual samples were performed in 10 µL reaction mixtures which contained 4 µL cDNA (10 ng), 0.5 µL of 2.5 µM each forward and reverse primers, 5 µL of 2× Faststart SYBR Green master mix (Roche, Germany) for the sample (+RT), 10 ng total RNA for negative reverse transcriptase (-RT) and PCR grade water for non template control (NTC), respectively. The reaction was performed in Light Cycler®96 (Roche, Germany) LightCycler® 480 Multiwell clear plate 96 (Roche, Germany). The PCR cycling condition was 95°C for 3 min initial denaturation followed by 50 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 15 sec. The melt curve analysis condition was 1 cycle 1 min initial denaturation at 95°C, 1 min annealing at 55°C and melting at 55–95°C for 10 sec. The relative mRNA abundance of target genes in tissue samples was determined

Table 2. Selenoprotein and endogenous control genes with their primers properties used in qPCR analysis

Gene name	Gene synonyms	NCBI Accession No.	Primer sequence (5′–3′)	Length (bp)	Tm (°C)	Amplicon size (bp)
Glutathione peroxidase 1 (GPX1)	GPx1; GPXD; GSHPX1	XM_004018462	GCTCTCCGGGGATTTTGCC	19	55.4	127
			TGTGGTCTGGGAAAGGGGA	19	53.3	
Glutathione peroxidase 2 (GPX2)	GPx2; GSHPX2	XM_004010720	CCTGGATGGGGAGAAGGTAGA	21	56.3	73
			AAGCGAGGCCACATTCTCAAT	21	52.4	
Glutathione peroxidase 3 (GPX3)	GPx3; GPX3	XM_004008983	CAGGAAGAGCTTGAACCATT	22	54.8	114
			CGTCGAACATACTTGAGGGTGGC	21	54.4	
Thioiredoxin reductase 1 (TXNRD1)	TR1, TrxR1	XM_004006684	CTGAAGGCTTCTCAGGAAAGC	21	54.4	86
			AGGTCGTAGTCATAGGGCTCC	21	54.4	
Thioredoxin reductase 3 (TXNRD3)	TR3; TRXR3	XM_004018720	CATCCTAGCTGCGTCTGGTC	20	55.9	168
			CCTCTGGTTGGTCATCTCCG	20	55.9	
Iodo thyronine deiodinase 1 (DIO1)	ITDI1; TXDI1	XM_004001999	ATCCCTACCTTCTTCAGTGCC	21	54.4	154
			CTTGCATGAAGTCCCAGATGC	21	54.4	
Iodo thyronine deiodinase 2 (DIO2)	ITDI2; TXDI2; SelY	XM_004011089	GCGTAGACTTGCTGATCACAC	21	54.4	97
			ACGTGCTTGAGGAGAATGACC		54.4	
Iodo thyronine deiodinase 3 (DIO3)	TXDI3; Type 3 DI; 5DIII; DIOIII	NM_001122650	GCGCCTAACTCTGAGGTGG	19	55.4	167
			TCGCGCTGATACTTGGTGAC	20	53.8	
15 kDa selenoprotein like protein (SEP15)	Selenoprotein F (15 kDa)	XM_004002164	AGCACAGCCCATGATAGGTAAG	22	54.8	107
			CCACAAGTCTACTACCAGGCATT	24	57.4	
Selenoprotein P1 (SEPP1)	SeP; SELP	XM_004017013	CGGAAGGGGTTCTGACAAC	19	53.3	89
			GCTCTCTGTTCCTCCGGTTAG	21	56.3	
Selenoprotein W (SEPW1)	SEPW1; Sep W; SEL W	XM_004023205	TGTTTACTATGGCGCTTGAGG	21	52.4	141
			CCCGCTACGAACACTTCAAAG	21	54.4	
Gyceraldehyde -3-phosphate dehydrogenase	GAPDH	NM 001190390.1	AGGAGCACGAGAGGAAGAGAG	21	60	101
			TGAGGATGGAAATGTATGGAG		60	

by the method of Livak and Schmittgen (2001). PCR reactions of each sample were conducted in triplicate.

Histology of tissues: After sacrifice, gross examination was performed. The representative muscle tissue samples (n=4) collected in 10% formalin were fixed for 48 h and washed in running tap water for 1 h. Fixed tissues were dehydrated with increasing concentrations of ethanol (70, 90 and 100%; each for 1 h) and cleared in xylene for 1 h for two changes. Tissues were kept in melted paraffin at 56ºC for three changes to embed in paraffin. Longitudinal and transverse sections (5µm) were prepared with semiautomatic microtome and placed on glass slide coated with Meyer's egg albumin. Tissue sections were dried by incubating them for 2 h at 40ºC and fixed sections were rehydrated in decreasing grades of alcohol (100, 90, 70 and 50%; each for 1 h) and then water. The sections were stained with haematoxylin and eosin stain (Bancroft and Stevens 1996) with some modifications, were covered with DPX (SRL, India) mounting medium with cover glass and observed under light microscope (Nikon, Japan) to study the histopathological changes.

Statistical analyses: All the experimental data generated were analyzed using the Statistical Package for Social Sciences (SPSS, version 18.0). The differences in antioxidant, lipid peroxidation and mRNA expression status between the control and Se-supplemented groups was determined by one-way analysis of variance (ANOVA) followed by Duncan posthoc tests for antioxidant, lipid peroxidation and LSD for gene expression status. The means of different groups were considered significant at P≤0.05 and P≤0.1. Results are presented as means±SE.

RESULTS AND DISCUSSION

The total antioxidant capacity in control and 0.5 ppm Se supplemented lambs showed no significant difference, whereas the lambs supplemented with 1.5 and 4.5 ppm Se showed increased (P<0.001) FRAP values as compared to the control (Table 3). The ferric reducing antioxidant power (FRAP) assay provides the basic information on the antioxidant status of biological samples. It determines the reduction of ferric ions to the ferrous form in which the electron is donated by antioxidants (Khajali *et al*. 2010). In the present study, the higher FRAP observed in *Longissimus dorsi* muscles of lambs supplemented with Se indicated the improvement of antioxidant status in the LD muscle tissues. In accordance with our results, supplementation of organically bound selenium increased FRAP and thus their antioxidant capacity in postpartum sows (Horky *et al*. 2013). Similarly, Khajali *et al*. (2010) reported a similar, but nonsignificant increase of antioxidant capacity in broilers supplemented with Se. The improved antioxidant capacity of meat measured by FRAP indicates its availability to customers as it is not affected by high temperature cooking (Serpen *et al*. 2012).

Table 4 shows the meat MDA concentrations in lambs offered diets supplemented with 0.5, 1.5 and 4.5 ppm Se as Se yeast or without Se. On day 0, the malondialdehdye levels in meat of Se supplemented lambs were significantly (P<0.002) low compared to control. However, on day 3 and day 7 of storage, there was no significant difference among the different treatments. Malondialdehdye is a major product of lipid peroxidation and quantification of MDA is a well-accepted method for determining the lipid oxidation of meat (Botsoglou *et al*. 1994). Vacuum packaging (VP) is one of the most common packaging methods for storage of fresh meat. The reduction in lipid peroxidation in meat samples of Se supplemented lambs on day zero observed in the present study could be due to the antioxidant effect of selenium. However, present study revealed that Se has limited potential to improve oxidative stability of meat during storage for longer periods as the concentrations of lipid peroxidation products were not reduced further by Se supplementation after 3 and 7 days of storage. Consistent with present study, Juniper *et al*. (2008) and Vignola *et al*. (2009) did not find any improvement in oxidative stability of lamb meat by feeding Se yeast at the dose level of 0.35 ppm for 112 days and 0.45 ppm for 63 days, respectively. It is also worth to reason that during storage, Se as an antioxidant, may be more effective in improving the oxidative stability of broiler meat (Oliveira *et al*. 2014) and pigs (Calvo *et al*. 2017), that are rich in unsaturated fatty acids than ruminant meat which contain more of saturated fatty acids. The science involved in such mechanism needs to be unveiled. However, supranutritional Se did not negatively impact lipid peroxidation and in turn the acceptability or marketability of lamb meat during storage since the MDA levels in meat of all the lambs up to seven

Table 3. Total antioxidant activity (Mean±SE) in the meat of lambs supplemented with varying levels of Se for 90 days

Group	Control	Se-0.5	Se-1.5	Se-4.5
Levels of Selenium (ppm)	0	0.5	1.5	4.5
FRAP (as mg of gallic acid equivalents (GAE)/g of muscle)	9.6±0.3^{y}	9.7±0.2^{y}	11.0±0.2^{x}	10.9±0.1^{x}

$^{x, y}$Values (Mean±SE) in the rows bearing different superscripts are significantly different (P<0.001).

Table 4. Meat MDA concentrations (Mean±SE) of lambs supplemented with varying levels of Se for 90 days

Treatment (n=5)	Selenium (Se) level (ppm)	Storage time (days)		
		Day 0	Day 3	Day 7
Control	0	0.40±0.03^{x}	0.36±0.03^{x,y}	0.54±0.04^{x,y}
Se-0.5	0.5	0.30±0.01^{y}	0.44±0.02^{x}	0.63±0.07^{x}
Se-1.5	1.5	0.28±0.02^{y}	0.31±0.03^{y}	0.46±0.01^{y}
Se-4.5	4.5	0.31±0.01^{y}	0.42±0.03^{x}	0.54±0.03 ^{x,y}
P values		0.002	0.015	0.114

^{x,y}Values (Mean±SE) with different superscripts in a column differ significantly (P<0.05).

days storage were within the acceptable limits of consumers.

A number of studies have shown that mRNA expressions of selenoproteins are influenced by Se supplementation (Zhou *et al*. 2009, Sunde and Raines 2011). However, to the best of our knowledge, this is the first report on influence of dietary supplementation of supranutritional levels of organic Se on selenoprotein mRNAs expressions in LD muscle tissues of growing lambs. The results showed that dietary Se at supranutritional levels influences mRNA expression of selenoproteins. Some selenoproteins are more sensitive to dietary supranutritional Se and such sensitivity differed with the type of selenoproteins and the concentration of Se.

The relative mRNA expressions of GPXs in lambs supplemented with different levels of Se are presented in Fig. 1. Compared to controls, the relative mRNA expressions of GPX1 did not differ in any of the groups supplemented with different levels of Se (C vs 0.5Se, P=0.486; C vs 1.5Se, P=0.419 and C vs 4.5Se, P=0.412). Compared to controls, GPX2 mRNA expressions did not differ significantly in lambs supplemented 0.5 ppm (P=0.74) and 1.5 ppm Se (P=0.375). However, GPX2 mRNA was significantly upregulated in lambs supplemented 4.5 ppm Se (P=0.063) as compared to controls and lambs supplemented 0.5 ppm Se (P=0.031). No significant difference in GPX3 mRNA expression was observed between controls and lambs supplemented 0.5 and 1.5 ppm Se (P=0.462 and 0.451 respectively). Conversely, GPX3 mRNA was significantly downregulated in lambs supplemented 4.5 ppm Se (P=0.023) compared to controls and lambs supplemented 0.5 ppm (P=0.004) and 1.5 ppm Se (P=0.004).

The differences in the mRNA expressions of three isoforms of GPXs namely GPX1, GPX2 and GPX3 by Se imply the possible involvement of isoform and

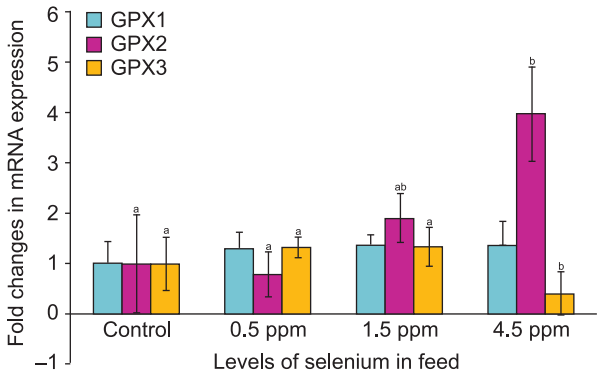

Fig. 1. Relative mRNA expression of the GPX genes in *Longissimus dorsi* muscle of lambs supplemented with varying levels of Se for 90 days, The fold change of expression was calculated using the formula of Livak and Schmittgen (2001) and normalized against the expression of control values. Values represent means±SE. Means with different superscripts $^{(a,b)}$ for a specific gene differ significantly. GPX1, Glutathione peroxidase 1; GPX2, Glutathione peroxidase 2; GPX3, Glutathione peroxidase 3.

concentration dependent regulatory mechanisms in their transcription. GPX1 is a key antioxidant selenoprotein of the GPX family expressed in all cells and its mRNA is a 13 kb transcript (Zhang *et al*. 2013a). In this study, we did not find difference in GPX1 mRNA expression in lambs fed with low and high Se. Similar findings are reported by Zhang *et al.* (2013a) in LD muscles of goats from a Se-enriched and Se-low regions. In a previous report, Hugejiletu *et al*. (2013) suggested that it might take more than one-year time for sheep GPX1 to be affected at mRNA levels. Therefore, the underlying possibility for the insensitivity of GPX1 in our study could be that the 90 day supplementation was not sufficient to affect GPX1 mRNA expression and we could have got some effects if the study had been continued further. On the other hand, the unaltered GPX2 mRNA expression in lambs supplemented with 0.5 and 1.5 ppm Se suggests that, GPX2, as a tissue specific isoform is predominantly expressed in gastrointestinal tract and less expressed in muscle tissue (Behne and Kyriakopoulos 2001). Consistent with present findings, Juszczuk-Kubiak *et al*. (2016) reported no changes in GPX2 mRNA levels in liver and skeletal muscles of lambs supplemented with 0.5 ppm inorganic Se to a diet containing 0.15 ppm Se. In contrast, GPX2 mRNA was upregulated in lambs supplemented with 4.5 ppm Se, suggesting that it could be stimulated in muscle tissue too, but with higher levels of Se. Such an upregulation of GPX2 mRNA is reported to mediate anti-inflammatory and anti-carcinogenic effects of various natural antioxidants (Stoytcheva and Berry 2009). A possible mechanism for the GPX2 upregulation by 4.5 ppm Se could be through the activation of Nrf2/keap signaling (nuclear factor erythroid related factor 2/Kelch-like ECH-associated Protein 1) pathway as suggested by Zhang *et al*. (2013a). Alternatively, it has also been proposed by Wingler *et al*. (1999) that the translation factor SelB regulates the amount of GPX2 mRNA expression through its affinity and stability towards the mRNA. GPX3 is a key antioxidant selenoprotein of the cellular antioxidant defense system. In contrast to our findings, Yao *et al*. (2014) reported upregulation of GPX3 mRNA in Se supplemented chicken muscles. However, GPX3 mRNA in LD muscles of goats fed Se low and Se rich diets (Zhang *et al*. 2013a) were similar as observed in lambs supplemented 0.5 and 1.5 ppm Se. Present study confirmed that LD muscle GPX3 mRNA in ruminants is less sensitive to high dietary Se but upto 1.5 ppm for 90 days, beyond which, its sensitive. Studies indicate a translational control of GPX3 (Zhang *et al*. 2013a) but the transcriptional regulation of GPX3 is largely uncharacterized. Hence with our data as basis, further downstream processes could be studied to characterize its transcriptional regulation.

The relative mRNA expression of TXNRD genes in lambs supplemented with different levels of Se are presented in Fig. 2. Compared to control, TXNRD1 transcripts were significantly (P=0.046) upregulated in lambs supplemented 0.5 ppm Se without any significant difference in lambs supplemented 1.5 ppm (P=0.515) and 4.5 ppm Se (P=0.768)

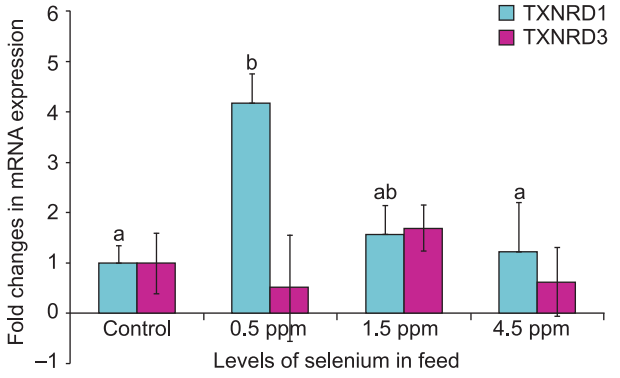

Fig. 2. Relative mRNA expression of the TXNRD genes in *Longissimus dorsi* muscle of lambs supplemented with varying levels of Se for 90 days, The fold change of expression was calculated using the formula of Livak and Schmittgen (2001) and normalized against the expression of control values. Values represent means±SE. [a,b]Means with different superscripts for a specific gene differ significantly. TXNRD1, Thioredoxin reductase 1; TXNRD3, Thioredoxin reductase 3.

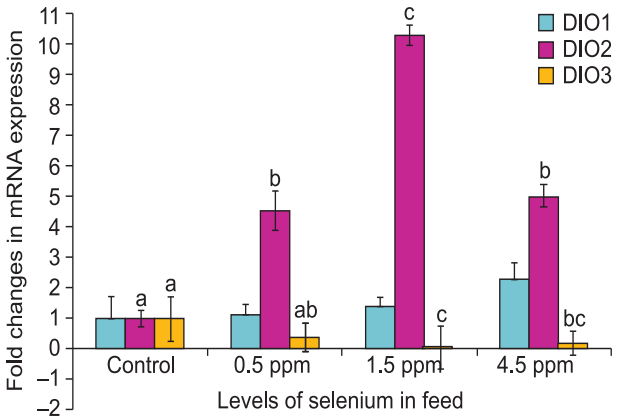

Fig. 3. Relative mRNA expression of DIO genes in *Longissimus dorsi* muscle of lambs supplemented with varying levels of Se for 90 days, The fold change of expression was calculated using the formula of Livak and Schmittgen (2001) and normalized against the expression of control values. Values represent means±SE. [a,b,c]Means with different superscripts for a specific gene differ significantly. DIO1, Iodothyronine dediodinase 1; DIO2, Iodothyronine dediodinase 2; DIO3, Iodothyronine dediodinase 3.

(Fig. 2). However, the TXNRD3 mRNA remained unaffected by the dietary selenium supplementation.

Similar to GPXs, the differences observed in mRNA expression of TXNRD1 and TXNRD3 genes suggests the involvement of independent mechanisms in their regulation by Se. Present results showed that 0.5 ppm Se had a significant effect on TXNRD1 expression and it is regarded as a potential biomarker for supranutritional Se status (Sunde and Raines 2011). TXNRD1 mRNA is regulated by Nrf2 and the mammalian thioredoxin reductase protein plays a major role in redox homoeostasis. Se is established to upregulate the other isoform TXNRD2 mRNA expression through an Nrf2 mediated mechanism that would translate into respective protein (Stoytcheva and Berry 2009) to regulate redox homeostasis of cells. However, it is not established if such mechanism exists to control the expression of TXNRD1 as well.

Fig. 3 shows the relative mRNA abundance of DIO genes in experimental animals. As compared to controls, the muscle DIO1 mRNA expression did not show any significant difference while DIO2 mRNA expression were significantly (P= 0.001) upregulated in all the three groups supplemented with Se. Further, DIO2 mRNA was found upregulated significantly in lambs fed 1.5 ppm Se as compared to lambs supplemented 0.5 ppm (P=0.049) and 4.5ppm Se (P=0.078). The expression of DIO3 mRNA was not altered by 0.5 ppm Se, however 1.5 ppm and 4.5 ppm Se supplementation resulted in downregulation (P=0.001 and P=0.008 respectively) of this gene as compared to control. In a previous report, supplementation of sodium selenite at 0.2 ppm concentration to a Se deficient diet upregulated DIO expression in skeletal muscle of chickens (Yao *et al*. 2014). Similarly, Se supplementation increased DIO1 and DIO2 transcripts in cultured ovine thyrocytes (Foroughi *et al*. 2013). In the present study, the expressions of DIO mRNA subtypes are independent of each other in terms of their response to Se supplementation. The differences observed in DIO2 and DIO3 mRNA expression patterns could be due to the different regulation mechanisms of both the isoforms. It has been reported that DIO2 transcription happens via cAMP dependant and DIO3 transcription through cAMP independent P38MAPK and ERK pathways, respectively (Foroughi *et al*. 2013). Since DIO2 regulates the transcription of various physiologically relevant genes in muscle (Marsili *et al*. 2011), the upregulation of DIO2 by supranutritional Se needs to be further studied in relation to muscle physiological functions.

Fig. 4 shows that supplementation of 0.5 and 1.5 ppm Se did not affect the expression of LD muscle SEPP1 mRNA levels (P=0.516 and 0.319 respectively) compared to control. However, the highest (4.5 ppm) Se feeding resulted in significant downregulation of this gene as compared to the control (P=0.003) and 0.5 Se ppm (P=0.001) and 4.5 ppm Se (P=0.04) supplemented groups. The expression of muscle SEP15 did not differ significantly between control and lambs supplemented 0.5 ppm Se (P=0.821); however, this gene was significantly downregulated by feeding 1.5 and 4.5 ppm Se (P=0.021 and 0.001, respectively). Feeding 0.5 and 4.5 ppm Se did not influence SEPW1 mRNA expression while feeding 1.5 ppm Se significantly (P=0.043) downregulated this gene as compared to control.

The SEPP1 has 10 to 12 selenocysteine residues and is the major Se transport protein. During normal metabolism, dietary-derived Se absorbed in blood is taken up by the liver and used for the synthesis of SEPP1 which is then transported to the distant tissues. The Se from SEPP1 is taken up by the skeletal muscles via ApoER2 receptor mediated process and utilized locally (Burk and Hill 2015).

Supplementation of sodium selenite at 0.2 and 0.5 ppm are reported to upregulate SEPP1 in muscles of lambs (Juscz-Kubiak *et al*. 2016) and broilers (Yao *et al*. 2014). The exact mechanism responsible for observed effect of Se on SEP P1 mRNA expression in our study is not known. However there could be an involvement of miRNA mediated mechanisms as suggested by Dewing *et al*. (2012) or insufficient receptor concentrations in the control of SEPP1 transcription in muscle which needs to be studied further. In contrast to our findings, mRNA expressions of SEPW1 and SEP15 were upregulated by dietary Se supplementation in chicken jejunum (Zhang *et al*. 2013b, Liu *et al*. 2014) and lamb muscles (Jusczuk-Kubiak *et al*. 2016) respectively. However leukocyte SEPW mRNA expression was not altered by supplementation of 300 µg of Se-yeast /day for 48 weeks in humans (Hawkes *et al*. 2013). The difference observed could be due to the differences in dietary Se concentration, treatment time, the type of tissue and species studied. Information on the regulation of SEP15 is limited and hence the present findings could be baseline information for the future studies.

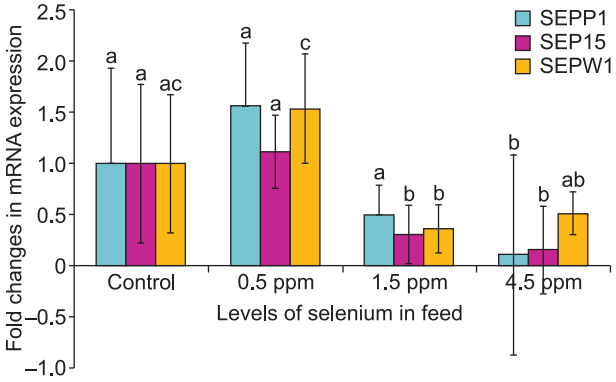

Fig. 4. The relative mRNA expression of SEPP1, SEP15 and SEPW1 genes in the *Longissimus dorsi* muscle of lambs supplemented with varying levels of Se for 90 days, The fold change of expression was calculated using the formula of Livak and Schmittgen (2001) and normalized against the expression of control values. Values represent means±SE. [a,b,c]Means with different superscripts for a specific gene differ significantly. SEPP1, Selenoprotein P; SEP15, 15 kDa selenoprotein; SEPW1, Selenoprotein W.

The present results reveal the highly controlled regulation of selenoprotein gene expression by dietary supranutritional Se. The differences in mRNA expression pattern amongst the different isoforms of GPXs, TXNRDs and DIOs reflect their sensitivity and differential regulation with changes in Se concentrations. Furthermore, amongst the Se sensitive selenoproteins, a linear increase in SEP mRNA expression with increasing levels of dietary Se was not observed, implying the involvement of intricate regulatory mechanisms in SEP gene expression based on

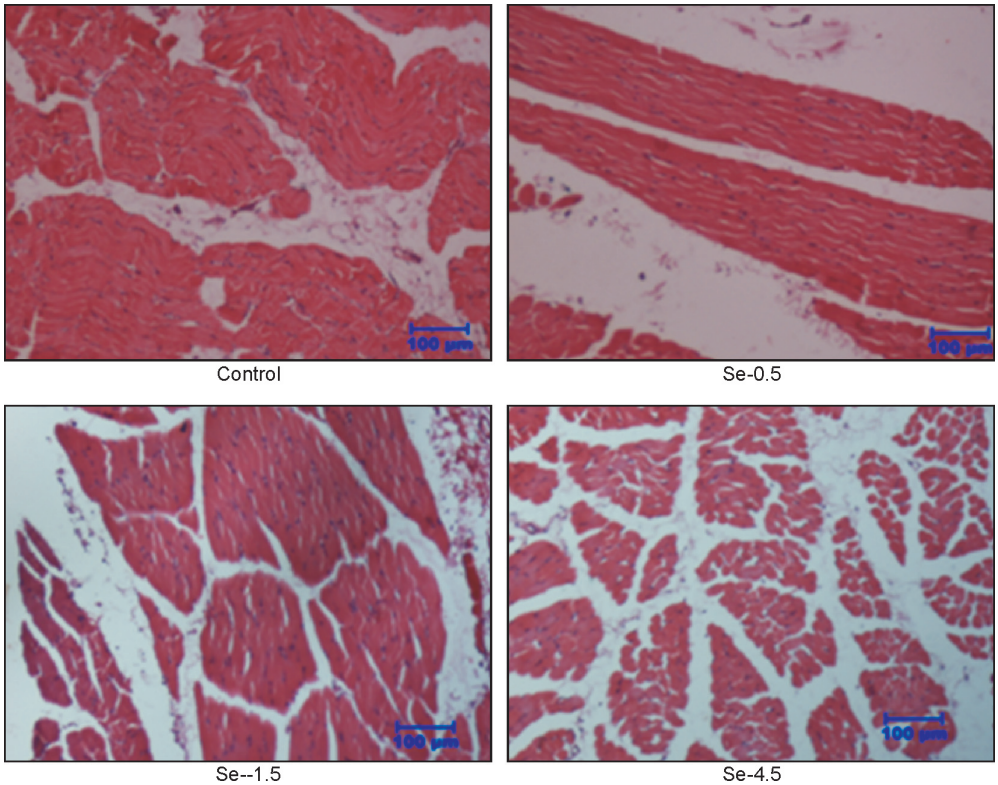

Fig. 5. Histology of *Longissimus dorsi* muscles of lambs supplemented with 0.5, 1.5 and 4.5 ppm Se, as Se yeast or without Se (control) for a period of 90 days. Control: Sheep fed basal diet without Se shows smooth muscle fibres with round blue staining nucleus, separated by adipose tissue layer with fat cells; Se-0.5, Se-1.5, and Se-4.5 groups: Sheep show normal architecture of muscle tissues similar to that of controls indicating no adverse effect of feeding organic selenium up to 4.5 ppm for 90 days to lambs. Haematoxylin and Eosin staining with scale bar=100 µm.

Se status. Selenoproteins are hierarchically classified as essential, viz. TXNRD1, TXNRD2, TXNRD3, DIO3, SEPS1 and SEPP1 and non-essential, viz. GPX1, GPX2, GPX3, DIO1, DIO2. It is suggested that the essential selenoproteins are preferentially supplied with Se at the cost of the non-essential selenoproteins during Se deficiency conditions (Carlson *et al*. 2009, McCann and Ames 2011). However, there are also reports wherein such hierarchy was not always observed (Sunde *et al*. 2009, Hujejiletu *et al*. 2013). On the other hand, the differences in mRNA expression patterns of the isoforms of GPXs, TXNRDs and DIOs, the SEPP1, SEPW1 and SEP15 in our study suggest that there could be a hierarchy in Se supply and selenoprotein gene function when Se is fed at different supranutritional levels.

Gross examination of LD muscles of experimental lambs showed no lesions. Microscopic observations of LD muscle tissues of control and Se supplemented lambs showed normal architecture without any adverse changes (Fig. 5). The muscle showed smooth muscle fibres with round blue staining nucleus separated by adipose tissue layer with fat cells.

Se exhibits a narrow range between deficiency and toxicity and hence histopathology of muscle tissues was studied. In line with the current findings, no gross and pathological changes were reported in tissues of lambs fed diet containing 10 ppm inorganic Se for 1 year (Cristaldi *et al*. 2005). Similar results were reported by Davis *et al*. (2008) in two year old sheep supplemented with up to 40 ppm dietary Se for 60 weeks. Further, we did not observe any symptoms of Se toxicity such as hair loss, fragility of fingernails or hooves, gastrointestinal upsets, skin rashes and/ or unpleasant garlic odour in exhaled air (Kieliszek and Bazejak 2016) in the experimental lambs. Present results thus indicate that Se supplementation upto 4.5 ppm for 90 days is safe and is of significance since *LD* muscle constitutes the major edible tissue for the consumers.

In conclusion, our results indicated that supranutritional dietary Se in sheep improved the antioxidant status of meat, reduced lipid peroxidation on day zero with limited or no improvement on oxidative stability of meat during storage. In addition, supplementation of up to 4.5 ppm Se did not induce any histopathological changes in muscles indicating its non-toxic effect on muscle. The 90 days supranutritional Se supplementation did not influence the expressions of GPX1, TXNRD3 and DIO1 but regulated GPX2, GPX3, TXNRD1, DIO2, DIO3, SEPP1, SEP15 and SEPW1 genes, indicating differential regulation of different selenoproteins in skeletal muscle. Our results thus provided new insights into the regulation of selenoprotein gene expression by supranutritional levels of dietary Se. Future studies are warranted to understand the effect of dietary supranutritional Se on the translational regulation of selenoproteins and metabolic pathways involving selenoproteins.

ACKNOWLEDGEMENTS

The authors thank Director, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, Karnataka, India for providing necessary facilities to carry out this study. Junior Research Fellowship to the first author provided by ICAR- NDRI, Haryana, is duly acknowledged.

REFERENCES

Bancroft J D and Stevens A.1996. *Theory and Practical of Histological Staining Techniques.* (4th edn). London, Churchill Livingstone.

Behne D and Kyriakopoulos A. 2001. Mammalian selenium-containing proteins. *Annual Review of Nutrition* **21**: 457–73.

Benzie I F and Strain J J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power, the FRAP assay. *Analytical Biochemistry* **239**: 70–76.

Botsoglou N A, Fletouris D J and Papageorgiou G E. 1994. Rapid, sensitive and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food and feed stuff samples. *Journal of Agricultural and Food Chemistry* **42**: 1931–37.

Burk R F and Hill K E. 2015. Regulation of selenium metabolism and transport. *Annual Review of Nutrition* **35**: 109–34.

Calvo L, Toldra F, Rodriguez A I, Lopez-Bote C and Rey A I. 2017. Effect of dietary selenium source (organic vs. mineral) and muscle pH on meat quality characteristics of pigs. *Food Science and Nutrition* **5**(1): 1–9.

Carlson B A, Yoo M H, Tsuji P A, Gladyshev V N and Hatfield D L. 2009. Mouse models targeting selenocysteine tRNA expression for elucidating the role of selenoproteins in health and development. *Molecules* **14**: 3509–27.

Cristaldi L A, McDowell L R, Buergelt C D, Davis P A, Wilkinson N S and Matin F G. 2005. Tolerance of inorganic selenium in wether sheep. *Small Ruminant Research* **56**: 205–13.

Davis P A, McDowell L R, Wilkinson N S, Buergelt C D, Alstyne R V, Weldon R N, Marshall T T and Matsuda-Fugisaki E Y. 2008. Comparative effects of various dietary levels of Se as sodium selenite or Se yeast on blood, wool, and tissue Se concentrations of wether sheep. *Small Ruminant Research* **74**: 149–58.

Dewing A S T, Rueli R H, Robles M J, Nguyen-Wu E D, Zeyda T, Berry M J and Bellinger F P. 2012. Expression and regulation of mouse selenoprotein P transcript variants differing in non-coding RNA. *RNA Biology* **9**: 1361–69.

Foroughi M A, Dehghani H, Mahdavi-Shahri N and Bassami M R. 2013. Sodium selenite increases the transcript levels of iodothyronine deiodinases I and II in ovine and bovine fetal thyrocytes *in vitro*. *Journal of Trace Elements in Medicine and Biology* **27**: 213–20.

Gandhi U P, Nagaraja T and Prabhu S K. 2013. Selenoproteins and their role in oxidative stress and inflammation. *Current Chemical Biology* **7**: 65–73.

Hawkes W C, Richter D and Alkan Z. 2013. Dietary selenium supplementation and whole blood gene expression in healthy North American Men. *Biological Trace Element Research* **155**: 201–08.

Hooper K J, Bobe G, Vorachek W R, Bishop-Stewart J K, Mosher W D, Pirelli G J, Kent M L and Hall J A. 2014. Effect of selenium yeast supplementation on naturally acquired parasitic infection in ewes. *Biological Trace Element Research* **161**: 308–17.

Horky P, Ruttkay-Nedecky B, Kremplova M, Krystofova O, Kensova R, Hynek D and Kizek R. 2013. Effect of different doses of organically bound selenium on antioxidant status and levels of metal ions in postpartum sows. *International Journal*

of Electrochemical Science **8**: 6162–79.

Hugejiletu H, Bobe G, Vorachek W R, Gorman M E, Mosher W D, Pirelli G J and Hall J A. 2013. Selenium supplementation alters gene expression profiles associated with innate immunity in whole-blood neutrophils of sheep. *Biological Trace Element Research* **154**: 28–44.

ICAR-NIANP. 2013. *Nutrient Requirement of Animals-Sheep, Goat and Rabbit*. pp 15–16.

Juniper D T, Phipps R H, Givens D I and Berin G. 2008. Tolerance of ruminant animals to high dose in-feed administration of a selenium-enriched yeast. *Journal of Animal Science* **86**: 197–204.

Juszczuk-Kubiak E, Bujko K, Cymer M, Wiciñska K, Gabryszuk M and Pierzchala M. 2016. Effect of inorganic dietary selenium supplementation on selenoprotein and lipid metabolism gene expression patterns in liver and loin muscle of growing lambs. *Biological Trace Element Research* **172**: 336–45.

Khajali F, Raei A, Aghaei A and Qujeq D. 2010. Evaluation of a dietary organic selenium supplement at different dietary protein concentrations on growth performance, body composition and antioxidative status of broilers reared under heat stress. *Asian Australasian Journal of Animal Science* **23**: 501–07.

Kieliszek M and Bazejak S. 2016. Current knowledge on the importance of selenium in food for living organisms: A Review. *Molecules* **21**: 609.

Kumar N, Garg A K, Dass R S, Chaturvedi V K, Mudgal V and Varshney V P. 2009. Selenium supplementation influences growth performance, antioxidant status and immune response in lambs. *Animal Feed Science and Technology* **153**: 77–87.

Liu C P, Fu J, Lin S L, Wang X S and Li S. 2014. Effects of dietary selenium deficiency on mRNA levels of twenty-one selenoprotein genes in the liver of layer chicken. *Biological Trace Element Research* **159**: 192–98.

Liu Y, Zhao H, Zhang Q, Tang J, Li K, Xia X J, Wang K N, Li K and Lei X G. 2012. Prolonged dietary selenium deficiency or excess does not globally affect selenoprotein gene expression and/or protein production in various tissues of pigs. *Journal of Nutrition* **142**: 1410–16.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene expression data using Real- Time Quantitative PCR and the and the 2-ΔΔCT Method. *Methods* **25**: 402–08.

Luciano G, Vasta V, Monahan F J, Lopez-andres P, Biondi L, Lanza M and Priolo A. 2011. Antioxidant status, colour stability and myoglobin resistance to oxidation of *Longissimus dorsi* muscle from lambs fed a tannin-containing diet. *Food Chemistry* **124**:1036–42.

Mariotti M, Ridge P G, Zhang Y, Lobanov A V, Pringle T H, Guigo R, Hatfield D L and Glladyshev V N. 2012. Composition and evolution of the vertebrate and mammalian selenoproteomes. *PLoS ONE* **7:** e33066.

Marsili A, Zavacki A M, Harney J W and Larsen P R. 2011. Physiological role and regulation of iodothyronine deiodinases: a 2011 update. *Journal of Endocrinological Investigation* **34**: 395–407.

McCann J C and Ames B N. 2011. Adaptive dysfunction of selenoproteins from the perspective of the triage theory: why modest selenium deficiency may increase risk of diseases of aging. *FASEB Journal* **25**: 1793–814.

Mehdi Y and Dufrasne I. 2016. Selenium in Cattle: A Review. *Molecules* **21**: 545.

Min B and Ahn D U. 2005. Mechanism of lipid peroxidation in meat and meat products -A Review. *Food Science and Biotechnology* **14**: 152–63.

Montossi F, Font-Furnols M, Campo M D, Julian R S, Brito G and Sanudo C. 2005. Sustainable sheep production and consumer preference trends: Compatibilities, contradictions, and unresolved dilemmas. *Meat Science* **95**: 772–89.

National Research Council. 2007. *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*. The National Academies Press, Washington, DC. https://doi.org/10.17226/11654.

Oliveira A F, Rodrigues S, Leite A, Paulos K, Pereira E and Teixeira A. 2014. Quality of ewe and goat meat cured product *mantas*. An approach to provide value added to culled animals. *Canadian Journal of Animal Science* **94**: 459–62.

Serpen A, Gokmen V and Fogliano V. 2012. Total antioxidant capacities of raw and cooked meats. *Meat Science* **90**: 60–65.

SPSS Version 18.0. 2010. SPSS Software products, Marketing Department, SPSS Inc. Chicago, IL, USA.

Stoytcheva Z R and Berry M J. 2009. Transcriptional regulation of mammalian selenoprotein expression. *Biochimica et Biophysica Acta-General Subjects* **1790**: 1429–40.

Sunde R A and Raines A M. 2011. Selenium regulation of the selenoprotein and non-selenoprotein transcriptomes in rodents. *Advances in Nutrition* **2**: 138–50.

Sunde R A, Raines A M, Barnes K M and Evenson J K. 2009. Selenium status highly regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome. *Bioscience Reports* **29**: 329–38.

Vignola G, Lambertini L, Mazzone G, Giammarco M, Tassinari M, Martelli G and Bertin G. 2009. Effects of selenium source and level of supplementation on the performance and meat quality of lambs. *Meat Science* **8**1: 678–85.

Weiss W P and Hogan J S. 2005. Effect of selenium source on selenium status, neutrophil function, and response to intramammary endotoxin challenge of dairy cows. *Journal of Dairy Science* **88**: 4366–74.

Wingler K, Boecher M, Flohe L, Kollmus H and Brigelius-Flohe R. 1999. mRNA stability and selenocysteine insertion sequence efficiency rank gastrointestinal glutathione peroxidase high in the hierarchy of selenoproteins. *European Journal of Biochemistry* **249**: 149–57.

Yao H, Zhao W, Zhao X, Fan R, Khoso P A, Zhang Z, Liu W and Xu S. 2014. Selenium deficiency mainly influences the gene expressions of antioxidative selenoproteins in chicken muscles. *Biological Trace Element Research* **161**: 318–27.

Zhang L, Zhou Z, Li G and Fu M. 2013a. The effect of deposition Se on the mRNA expression levels of GPxs in goats from a Se-enriched county of China. *Biological Trace Element Research* **156**: 111–23.

Zhang Q, Liu B, Zheng L and Tang Z. 2013b. Effects of selenium overexposure on glutathione peroxidase and selenoprotein *W* gene expression in the jejunum. *Journal of Animal and Veterinary Advances* **12**: 1628–31.

Zhou J C, Zhao H, Li J G, Xia X, Wang K N, Zhang Y J, Liu Y, Zhao Y and Lei X G. 2009. Selenoprotein gene expression in thyroid and pituitary of young pigs is not affected by dietary selenium deficiency or excess. *Journal of Nutrition* **139**: 1061–66.