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Effects of Copper Supplementation on Lipid Oxidation and Meat Quality of Merino X Texel Lambs

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Abstract Two levels of copper sulphate and copper-methionine were evaluated on lipid oxidative stability in liver and *Longissimus thoracis* (LT) muscle, measured by thiobarbituric acid reactive substances (TBARS) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in liver. 40 Merino x Texel lambs were randomly distributed into 5 treatments with 8 animals each. Treatments were: control, without copper addition; 10 or 30 mg of Cu/Kg DM complete diet in the form of Cu-sulphate or Cu-methionine. After 120 days period, animals were slaughtered and samples collected, for analyses on activities of SOD, GPx and TBARS in livers. LT muscle was collected for TBARS analysis at carcass dressing, 3 and 6 days after chilling at 4°C and 12 months after vacuum freezing. SOD and GPx activities were higher in animals receiving copper. TBARS values in muscle were higher in the control group at the time of boning but did not differ after 12 months of vacuum freezing. TBARS values in muscle during shelf life, with a linear increase over the days, did not vary. It was found that copper supplementation, even at considered toxic levels of 30mg/kg DM, plus 9mg/kg basal diet, resulted in increased hepatic concentration of liver antioxidant enzymes and showed no detrimental effects on muscle oxidative stability.

Keywords: *copper supplementation, fatty acids, lipid oxidation, lamb meat*

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1. Introduction

Consumer demand for high quality food is determined by the crossover of several demanding threats challenging us today, as climate changes, biodiversity crisis and reducing waste, impact deeply our food systems. The global food system is the principal element of this shift on biodiversity degradation on the past decades due to considerable increase in food production based on high amounts of external-inputs, such as inorganic fertilizers, pesticides, and other amendments, under the 'cheaper food' paradigm [1].

High intakes of macronutrients is associated with oxidative stress onset, sparking inflammatory cascade, thus diet type contribute, through an intricate system, in the arbitration of inevitable oxidative stress and can function to intensify or reduce the level of inflammation [2].

One of main challenges in meat industry is to offer products with pleasant colour and flavour and these characteristics of freshness to be maintained throughout shelf life with minimum costs possible. Lipid oxidation is in the origin of the main organoleptic traits affecting consumer acceptance [3]. Lamb and mutton meat despite devoid of a high level content in unsaturated lipids, are nevertheless susceptible to lipid oxidation mainly in the presence of metal ions [4].

Around the world, with more than 1,000 different sheep breeds, there are many of domesticated sheep which produce meat, wool, skin and milk, but not all of them are bred for meat production. Choosing multi-purpose breeds or crossbreds for producing profitable market lambs depends on many factors, namely the production systems. Supplementary feeding of sheep and supplementation with micronutrients aims at preventing extensive live weight loss and improve carcass traits [5].

Meat or skeletal muscle represents some 35-60% of body weight and is characterized by muscle fibres, connective tissues (aponeuroses, ligaments, tendons), blood vessels, adipose and nervous tissues. These elements, namely marbling (intramuscular) fat and fatty acids, play key roles in the determination of nutritive meat quality [6].

After an animal is harvested for meat, anaerobic conditions are steadily established in muscle tissue. Anaerobic metabolism at the cellular level occurs when

oxygen transport and tissue oxygenation are compromised, increasing glycogenolysis and anaerobic glycolysis with ATP production, creatine phosphate conversion, augmenting the rate-limiting enzyme phosphofructokinase, and reducing aerobic synthesis by residual oxygen in the striated muscle myoglobin [7].

Organoleptic characteristics are very complex issues and important to determine quality and consumer preference. A growing number of applications are being developed for the improvement of food safety and quality as consumer choices now demand more, better, healthy and safer products [8]. Consumers rely on the visual appearance of meat products to indicate its freshness. Regrettably animal products can potentially be a source of biological and chemical contamination for consumers therefore research has to adapt itself to meet this challenge, an important issue in terms of public health [9].

The concept of food quality is becoming more sophisticated and increasingly applied in developed but heterogeneous markets, while in informal sector households and markets is arduous to implement [10]. Nevertheless, lifestyle and food preferences based on cultural differences helps to maintain the mosaic of farming and food production practices. For example, there is a detectable sensory difference in sheep meat produced in different regions of southern Africa [11].

1.1. Lipid Oxidation

Meat and meat-products are susceptible to degradation processes, microbiological and oxidation, and while lipid peroxidation is well known for decades, their detailed theoretical and practical mechanisms are not yet completely perceived [12]. Meat products are liable to polyunsaturated fatty acid oxidation in the presence of oxygen, or exposed to light, heat, free radicals, or mixed with some additives. Processing strategies and techniques, such as temperature control, heating, and packaging, can also influence the oxidation of meat products [13].

The demand for quality food is increasing, but meat that has been oxidized reduces gradually the sensory and nutritional quality due to the loss of essential fatty acids and vitamins and may develop multiple toxic compounds, rancid aromas and flavours [14]. Taints and off-flavours are a major problem for the food industry. The lipid fraction degradation and oxidation contribute to organoleptic properties (e.g. aroma, off-odours, colour, texture and succulence), also impacting on protein stability, energy content and shelf-life [15].

Protein oxidation is also an important oxidative process impacting meat quality but it is the complex degradation of fatty acids into products such as hydro peroxides and lipid derived volatile aldehydes that are dominant and can occur through auto-oxidation by free radicals, photo-oxidation, enzymatic oxidation and thermal oxidation [16].

The adipose tissue is no longer regarded an inert body reserve of energy to be mobilized, instead is presently viewed as a substantial, active and powerful endocrine tissue in for metabolic homeostasis, producing leptin, protein factors and bioactive hormones adipokines [17,18]. The enzymes that participate in fatty acid catabolism are located in the mitochondria, cytoplasmic organelles considered the energy power station of cells. Brown fat, contrary to white fat which only contains residual mitochondria, is a metabolically active tissue rich in mitochondria transferring, by dissipation, chemical energy from food as heat [19].

Lipid oxidation may be affected by many ante- and post-mortem factors, the presence and concentrations of pro- or antioxidants, the handling, transport and storage [20,21].

1.2. Copper Supplementation

There is a double-dealing on microelement nutrients, including copper, as there is a risk of nutrient deficiency or excess, so they may be beneficial or harmful, being essential elements but potentially toxic [22].

Copper has been used as an affordable and successful growth promoter in several animal species since the 60's but its overuse not only may harm the health of livestock but can also affect the environment, food safety, public health and the economy [23]. Copper is an essential trace metal element in animal and human metabolism and health but unnecessary and persistent amounts are toxic, causing cellular damage and disruptions in physiologic homeostasis [24].

Copper supplementation in sheep deserves special attention because of the narrow margin between deficiency and toxicity, both of which sheep are susceptible to suffering from. Proposed copper levels in the lamb diet are about 10 mg/kg DM, with maximum permitted levels to avoid toxicity being 17 mg/kg DM [25].

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), developed an algorithm to derive newly proposed maximum contents from the requirement and the native dietary copper content. These values (mg Cu/kg complete feed) are depicted in Table 1.

Table 1. Maximum permitted levels of copper exist in the EU to protect both animal and human health

EU Regulations		
EU 2018 regulations on the maximum permitted level of copper in a complete feed were updated (sheep and young stock remained the same) to the following:		
Animal	**Old maximum permitted level**	**New maximum permitted level**
	Fresh weight (dry matter shown in brackets) mg/kg	Fresh weight (dry matter shown in brackets) mg/kg
Cattle (beef and dairy)	35 (40)	30 (34)
Calves pre rumination (beef and dairy)	15 (17)	15 (17)
Sheep	15 (17)	15 (17)
Goats	25 (27,5)	35 (40)

If copper homeostasis is disrupted or maximum recommend levels are surpassed, copper may rapidly accumulate in hepatocytes causing, over a lengthy period, chronic toxicity cases [26].

The greater part of copper absorbed from the gastrointestinal tract actually arises from endogenous fluid secretions on a permanent recycling event. Ceruloplasmin, albumin and α-2-macroglobulin carries some 95% of copper found in the blood serum and excess copper is released from liver into the bloodstream [27].

In healthy humans and animals copper binds to the non-polypeptide component of enzymes or is firmly linked to copper transport and copper chaperones necessary for the delivery of copper to specific cuproenzymes such as superoxide dismutase (SOD) and cytochrome c oxidase (COX) (Figure 1).

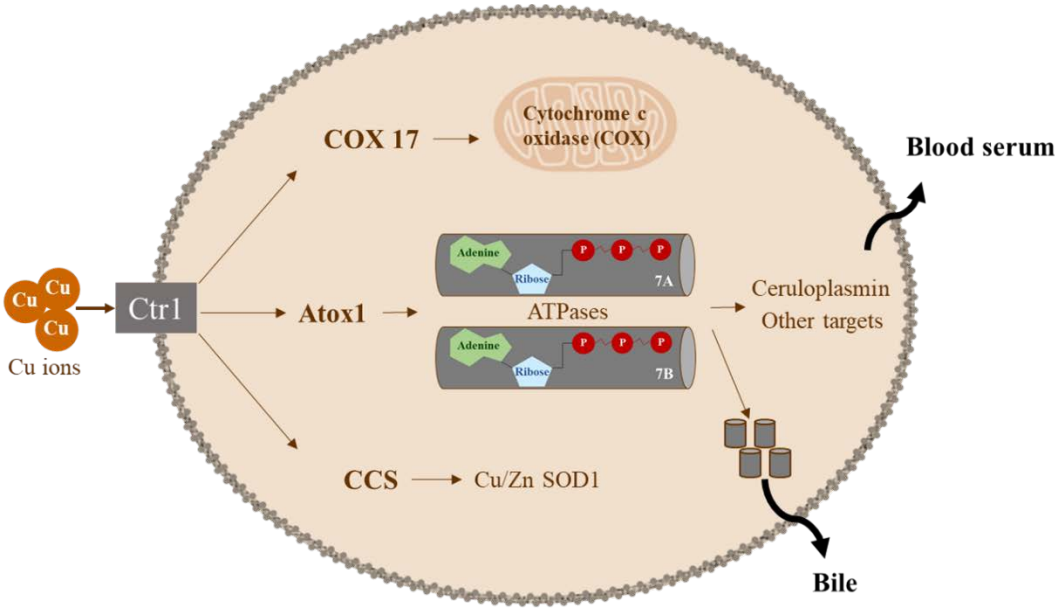

Figure 1. Some 70% of copper is taken up at the brush border in intestinal cells by a specific copper ions transporter (Ctr1). These enter the cytoplasm and follow 3 different pathways mediated by 3 different Cu chaperones in the cytoplasm: COX17 (an essential protein in the assembly of cytochrome c oxidase within the mitochondrion), Atox1 (binds and deliver cytosolic copper to the copper ATPase proteins) and CCS (delivers Cu to superoxide dismutase)

The ATP-driven transporters, such as copper-transporting Golgi-localized ATPases (Cu-ATPases) ATP7A and ATP7B, play a central role in this process catalysing the translocation of copper across cellular membranes [28].

Copper is a cofactor with an essential role for life, and a structural component of various metalloenzymes [29]. It is involved in important biochemical pathways such as the respiratory chain, antioxidative defence, and iron metabolism [30]. However, much remains to be discovered, in particular, how to regulate copper homeostasis to prevent neurodegeneration, when to chelate copper, and when to supplement it [31,32].

The addition of natural or synthetic antioxidants directly in meat [33] or used indirectly in animal diets provides varying effects in prolonging the shelf life of lamb meat [34] all encircling lipid oxidation [35].

Water scarcity, often combined with heat stress, is a common challenge facing these animals, and different lamb breeds have been studied for their acceptance and tolerance to copper [36,37], and have shown Merino breed more tolerant to dietary Cu than other sheep breeds [38]. Texel breed and crossbreds are less tolerant, and levels as low as 10 ppm Cu in a ration with nearly equal amounts of hay and concentrate may cause copper accumulation in liver [39,40].

The present trial, follows a previous one on different sources and levels of copper on lipid metabolism of lambs [41] and aims at studying the effects of diet supplementation of Merino x Texel lambs with two levels of copper sulphate and copper-methionine, on the stability of lipid oxidation in *Longissimus thoracis* (LT) muscle and liver, measured by thiobarbituric acid reactive substances (TBARS) and the activities of enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx**)** in livers.

1.2.1. SOD and GPx Enzymes

General protocols are described to measure the antioxidant enzyme activity of superoxide dismutase (SOD), glutathione peroxidase, catalase, or as total antioxidant activity. We have chosen the first two in the present studies as the main antioxidant enzymes, which are capable of stabilizing or deactivating free radicals before they oxidize cell components.

1.3. Copper Supplementation and Gut Microbiota

In ruminants, cattle, sheep and goats, high purity copper needles and bolus have been used since the 70's to supplement poor pastures and also for control of nematode parasites [42]. The rumen microbiota is fundamental for the productivity and health of sheep and copper supplementation is known to increase the diversity of the rumen microbiota [43].

Gut microbiota can change how animals metabolize copper as this trace nutrient is a critical enzyme cofactor in the body and a potent cellular toxin when intracellularly unbound to proteins. The role of the trillions of microorganisms that reside in the reticulo-rumen and large

intestine of ruminants, in copper metabolism is still not well understood but it is suggested a critical relationship between the intestinal microbiota and the metabolism of copper [44]. Copper absorption may be increased by using rumen-protected sources of this trace element, thereby reducing its excretion in the environment [45].

There is a need to optimise the type, dose and duration of Cu-supplementation for sheep, depending on their biological effects. Moreover, the bioavailability of Cu-chelating agents in sheep still needs further confirmation being among the most promising tools to keep copper concentration at physiological levels.

Copper sulphate is a readily available and widely used inorganic Cu source [46], but the efficiency of absorption in ruminants is very low for inorganic sources [47]. Copper methionine is recommended for the prevention and treatment of copper deficiency in sheep. The duration of the effect of a single dose will depend on the initial copper status of the animal; in most cases it will be effective for 2-4 months. Using a copper chelate supplement provides a prolonged rise in the copper status of the animal although inorganic form of Cu (copper sulphate) are considered more efficient than organic (copper chelate) in influencing the Cu metabolism in goat kids [48].

The present study aimed at assessing the effects of two forms of copper supplementation on frozen storage duration, quality characteristics, and lipid oxidation on a lamb muscle.

2. Materials and Methods

2.1. Trial Location

The 120-day trial was conducted at facilities of São Paulo University, Brazil.

2.2. Animals and Experimental Procedures

Forty lambs were chosen for similar average starting live weight (22kg ± 0,46 kg) from non-castrated Merino x Texel crossbred at fattening stage, selected and submitted to treatments till slaughter at 41,82 ± 0,51 kg.

Animals were kept individually in metabolic crates in a controlled room, with controlled drinking and feeding bowls.

At the onset faeces from all lambs were collected to evaluate parasitic contaminations and were subsequently dewormed with oxibendazole. They were submitted to a 40-day period of gradual diet adaptation. During this period they received basal experimental diet with no copper supplementation. All animals endured liver biopsies using the procedure described by Miles, Wilkinson e McDowell [46] at time zero during adaptation period.

Subsequently all lambs were intramuscular injected with antibiotic (Pencivet® PLUS PPU), 1 mL/10 kg live weight for 3 days, followed by a 15-day recovery period.

2.3. Treatments

Animals were randomly allocated in 5 groups of 8 lambs each. Treatments were:

(i) Control (basal diet containing 9 mg Cu/kg DM), without the addition of Cu (Co); **(ii)** 10 mg Cu/kg DM from Cu-sulphate ($CuSO_4$); **(iii)** 30 mg Cu/kg DM from $CuSO_4$; **(iv)** 10 mg Cu/kg DM from Cu-methionine, and **(v)** 30 mg Cu/kg DM from Cu-methionine.

Feeds were offered daily at 06:00 and 16:00 hours in two equal portions. Basal diet integrated maize, cottonseed hulls and soybean (Table 2) and was formulated to have 16% crude protein (CP) and a 65% total digestible nutrient (TDN). Refusals were weighed daily and sampled, and the quantity of feed provided for each lamb was adjusted weekly according to body weight. The use of only one grouped sample of orts per animal for the entire experimental period reduced labour and costs of laboratorial analysis. The experiment lasted for 80 days.

Table 2. Composition of the basal diet [a]

Ingredient	(%)
Ground corn	55.8
Cottonseed hulls	25.0
Soybean meal	16,0
Soybean oil	1,0
Calcitic limestone	1,2
Vitamin premix[b]	0,5
Mineral premix[c]	0,5
Total	100

[a] Dry matter basis and contained 9.0 mg of Cu/kg.
[b] Contained per kilogram of premix: 400,000 UI of vitamin A and 4000 UI of vitamin E.
[c] [c]Contained per kilogram of premix: 100 mg of Iodine, 4000 mg of Iron, 40 mg of Cobalt, 3000 mg of Manganese, 40 mg of Selenium, 4000 mg of Zinc, 40 g of Sulphur and 216 g of Sodium Chloride.

2.4. Slaughter and Sample Collection

Animals were slaughtered at the end of trail period with live weight 41,82 ± 0,51 kg after a full starvation period of 16 hours.

Liver samples were collected in triplicate at the abattoir, properly packed in aluminium foil and deep frozen in liquid nitrogen for subsequent analyses of superoxide dismutase (SOD), glutathione peroxidase (GP-x), thiobarbituric acid reactive substance (TBARS) and copper.

All carcasses were kept in a refrigerator chamber at 0ºC until inception of *rigor-mortis*. After 24h cooling, each left side of the carcass was parted between the 12th and 13th ribs, LT muscle samples (2.5 cm thickness) collected from 13th rib in cranial direction. From these samples 5 g were selected in triplicate, placed in aluminium foil, and immediately frozen in liquid nitrogen for subsequent TBARS and copper analyses. The left loin was totally removed from each carcass, vacuum freeze for later analysis of shelf life or display-life (DL).

2.4.1. Vacuum Freezing

Samples from each full loin of each carcass were vacuum packed in special plastic oxygen barrier film (200 mm x 310 mm, code B530FZ, Cryovac, Brazil), and frozen (0°C) for 12 months.

After this storage period, 5 g in triplicate of the LT muscle were selected and immediately kept in liquid nitrogen for TBARS 12 month's analysis.

2.4.2. Shelf Life or Display Life (DL)

After 12 months vacuum freeze, 3 samples from the LT muscle 2.5 cm thick were collected from each loin, individually placed in absorbent polystyrene trays and rapped with a polyvinyl chloride film and kept in a refrigerated display counter (vertical model, 125 LX, Auden, Brazil) at 4°C during 3 or 6 days. On these specific days, the tray from each animal was removed from the refrigerator and three sub-samples (5g each) were collected and frozen in liquid nitrogen for TBARS analysis (TBARS at 3 and 6 days defrost). Brazil has a long history on assessing food safety knowledge and practices of food handlers [49] and all guidelines were respected throughout the present trial.

2.5. Analytical Procedure

2.5.1. Lipid Oxidation

2.5.1.1. Thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was determined in liver and LT muscle by methodology proposed by Vyncke [90,96] with modifications. Thus, samples of 5g were homogenised with 15mL trichloroacetic acid (TCA) solution 7.5% (g/mL), containing EDTA 0.1% (g/mL) and propylgallate 0.1% (g/mL), for 30 seconds on a 25,000 rpm homogenizer (IKA, model Turrax T18) and filtered through paper filter Whatman grade 1.

The resulting filtrate was incubated at the ratio of 1:1 with thiobarbiturate (TBA) 0.02M in boiling water bath for 40 minutes. Test absorbance's were measured at 532 and 600nm wavelength in a spectrophotometer (Beckman Coulter, model DU800). The absorbance of the sample was considered as the difference between the considered wavelengths to correct any sample turbidity. Values were calculated from the standard curve with different dilutions of TEP (1,1,3,3-tetramethoxypropane) between 0,02 - 1,1 µg/mL and expressed as mg of malondialdehyde per kg sample.

2.5.2. Activities of Antioxidant Enzymes

2.5.2.1. Enzyme (SOD and GPx) Extraction

For extraction of enzymes SOD and GPx, liver samples were homogenised with sodium phosphate buffer solution (10 mM pH 7.4) at 1:10 (g/mL) ratio, in a homogenizer (IKA, model Turrax T18) for 60 seconds at 25,000 rpm. Subsequently, samples were centrifuged at 10,000 rpm (Eppendorf 5810R) for 10 minutes at 4°C and supernatants were used to determine SOD and GPx activities.

2.5.2.2. Superoxide Dismutase (SOD) (EC 1.15.1.1)

The activity of SOD enzyme was determined by an enzymatic method kit (Randox Laboratories, UK). This method uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan compound. The SOD activity is measured through the degree of inhibition of this reaction and was expressed in µmol per minute per milligram of protein ((µmol/min/mg of protein).

2.5.2.3. Glutathione Peroxidase (GPx) (EC 1.11.1.9)

The activity of GPx was determined by the Panglia and Valentine method [50], through the assay kit Ransel (Randox Laboratories, UK). This method is based on the decrease of NADPH concentration at 340nm, in the presence of H_2O_2. This activity was expressed in nmol/min/mg protein.

2.5.2.4. Protein Determination

The protein content of supernatant from liver samples was determined on a spectrophotometer (Beckman, model DU-800) following the method described by Bradford [51], using bovine serum albumin to build the standard curve.

2.6. Statistical Analysis

Statistical analysis of data was achieved by analysis of variance (ANOVA) for a completely randomized design, with 5 treatments, using the GLM procedure in SAS (SAS Institute, Inc., Carry, NC, USA). In case of significant results, mean averages were examined by contrast using Scheffé test. A 5% significance level was adopted.

Experimental design was completely random, with 8 repetitions per treatment. The experimental unit was the animal. Depending on the covariance structure that modulates the errors within the plot, the general linear model (GLM) the Standard Linear Model was used for analysis for copper in liver and LT muscle.

For TBARS across period of examination, it was used the mixed model (MIXED) of SAS. Averages were compared by contrast and a 5% level of significance adopted.

(i) In case main of significant effect of treatment, contrasts were evaluated by Scheffé test.

(ii) In case of main significant effect of day periods, it was performed a regression analysis aiming at evaluating the behaviour of the response variable questioning time period function after meat defrost for TBARS analysis.

(iii) In case of significant interaction, regression analysis was performed to evaluate the behavior of each treatment and time function.

3. Results and Discussion

3.1. Copper in Liver and LT Muscle

At the end of the experimental period, it was observed the otherwise expected major concentration of copper in livers of supplemented animals when compared to group control ($P<0.05$) (Table 3). As anticipated, group receiving 30mg/kg DM of Cu-methionine showed significantly ($P<0.05$) higher copper concentration compared to group given 10 mg/kg DM from the same source (Table 3).

Table 3. Effect of cooper source and level on liver and LT muscle cooper concentrations of Merino x Texel lambs

Variables	Treatments (mg Cu /kg DM)					SEM
	Co 0	CuSO4 10	CuSO4 30	Cu-met 10	Cu-met 30	
Initial Cu liver (mg/kg)	39.90	47.85	48.83	29.51	60.77	13.34
Final Cu liver (mg/kg)	168.01^{A}	247.86^{B}	284.95^{B}	263.02^{B}	341.29^{C}	23.33
Cu LT muscle	1.77	1.95	2.08	1.83	2.00	0.11

Co = Diet control (without supplemental copper).
SEM = standard error of the mean.
Significant differences are denoted with different superscripts (P<0.05).

Copper deficiency has long been known to alter lipid metabolism. The majority of absorbed dietary copper in the intestines is initially delivered to the liver where it deposits and is metabolized. Hepatocytes utilize copper for their metabolic needs and they also synthesize and secrete the major copper containing protein in serum, ceruloplasmin, and avoid copper excess in the body, thus in meat, by discharging surplus copper via the apical canalicular membrane of the hepatocyte into the bile [52].

Care must be taken when supplementing copper to a basal diet. This needs to have its ingredients controlled for copper content as, for example, in Asia it has been reported that the inclusion of palm kernel cakes in sheep rations greater than 80% for a long feeding time is associated with copper toxicity, which has a detrimental effect on the liver, especially in crossbreed sheep [53].

In relation to LT muscle, no differences were observed in copper concentration.

Copper supplementation in bovine and ovine diets, besides interacting with other minerals (e.g. sulphur, selenium and molybdenum), is a tough assignment and needs caution due to the slim limits between deficiency and toxicity, both of which these animals are prone to experience from. Sheep show higher sensitivity to copper when compared to other animals like goats or cattle. Warning of copper poisoning in sheep´s liver appear frequently in various publications and there is even the belief that copper should not be in lambs' diet [54].

Indeed, minerals have structural, metabolic, chemical reactor, and control functions in animals and when supplementing with copper in ovine diets, levels of this mineral in liver increase but not in muscles [55].

3.2. Antioxidant Enzymes

3.2.1. Superoxide Dismutase (SOD)

The supplementation of copper regardless of source or level, provided a greater activity of SOD in liver (P<0.05), when compared to group control. Animals supplemented with Cu-methionine showed greater activity of SOD when compared with those supplemented with $CuSO_4$ (P<0.05). There were no differences on copper supplementation among different levels within the same source (Table 4).

The increase in SOD activity in liver (P<0.05) followed the values obtained for copper concentration in this organ. This result supports the knowledge that SOD is an essential enzyme associated with copper in many tissues, inferring that diet supplementation increases its activity [56].

The increase in SOD activity in liver and blood after copper supplementation has been documented by other authors [57].

In reality, copper metabolism in sheep as specificities therefore reliable comparison should not conducted with other species, even goats. In sheep, copper metabolism is strongly affected by presence or absence of other minerals and ionophores. Molybdenum and sulphur act as antagonists to copper as they can bind to copper and affect rates of absorption and increased excretion from liver and other tissues [58].

3.2.2. Glutathione Peroxidase (GPx)

Activity values of GPx accompanied those of SOD. Copper supplementation, whatever the source or level, provided a greater activity of GPx in liver (P<0.05), when compared to group control.

Animals supplemented with Cu-methionine showed greater activity of GPx when compared with those supplemented with $CuSO_4$ (P<0.05). There were no differences on copper supplementation among different levels within the same source (Table 4).

Although the crucial role of copper in several enzymatic processes, this heavy metal can exert adverse toxicological effects, when present in high concentrations and bio-accumulate in livers. Several authors have documented a correlation between enzyme biomarkers and copper concentration [59].

According to several authors copper deficiency may reduce the activity of antioxidant enzymes including those non-dependent of copper such as catalase and selenium-GPx [60,61].

Numerous animal and human studies have demonstrated that copper deficiency can cause ischemic heart disease and that copper supplementation or adequate dietary copper can improve many of these risk factors [62]. Although also reported that GPx activity reduces in liver and plasma of pigs, rats and humans with copper deficiency [63], this may not necessarily be interpreted to be similar in sheep as metabolisms are quite distinct [64].

A complex antioxidant defence grid that relies on endogenous enzymatic (SOD, GPx, catalase) and non-enzymatic antioxidants is involved in body tissue metabolism with crucial role as scavenger of free radicals permanently generated within the mitochondria and preventing and treating oxidative stress [65,66].

Our results show a correlation between GPx and SOD activities, probable due to copper increase in liver which increases hydrogen peroxide (H_2O_2) thus increasing the synthesis of GPx, fundamental for the degradation of dispensable super oxide anion radical ($^{*}O_2$) which is perpetually generated in normal body metabolism through several processes [67].

Table 4. Activities of enzyme superoxide dismutase (SOD), in µmol/minute/mg of protein and glutathione peroxidase (GPx), in nmol/minute/mg of protein, in livers of Merino x Texel lambs receiving control diet or supplemented with copper from different sources and levels

Variables	Treatments (mg Cu /kg DM)					SEM
	Co	CuSO4	CuSO4	Cu-met	Cu-met	
	0	10	30	10	30	
SOD (µmol/mg prot)	22,41^{A}	30,42^{B}	31,36^{B}	37,86^{C}	38,31^{C}	1,27
GP-x (nmol/min/mg prot)	247,27^{A}	318,55^{B}	300,6^{B}	410,79^{C}	348,71^{C}	0,006

Co = Control diet (no supplemented copper).
SEM = standard error of the mean.
Significant differences are denoted with different superscripts (P<0.05).

3.3. Lipid Peroxidation

3.3.1. Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) are formed as products of fat degradation (lipid peroxidation) and its determination is used to evaluate lipid oxidation in meat as ROS are difficult to detect due to their very short half-lives.

Despite its limited analytical specificity and robustness, TBARS measures mainly malondialdehyde MDA) [68] and this assay has been extensively studied and used to try and link specific diseases and pathologic processes with the occurrence of free radicals [69].

Additionally, TBARS is used as an important freshness factor both in meat and fish to measure rancidity although fluctuations of TBARS values are known to occur during storage [70].

However, recent studies indicate that the use of peroxide value or TBARS as single indicators do not adequately characterize the extent of lipid peroxidation as it relates to animal performance, and may often provide misleading results [71].

TBARS values in liver and LT muscle are shown in Table 5. There were no differences in liver. Copper retained by hepatocytes is mostly bound to specific metal-binding proteins, primarily high cysteine content metallothionein, or incorporated into several cuproenzymes [72,73].

This provides a temporary storage for cytoplasmic copper, preventing it from occurring as (potentially toxic) free ionic metal and the link with proteins may inactivate their antioxidant activity, although this will vary with many factors such as genetics, nutrition and environment [74].

Lipid peroxidation in liver induced by copper is usually accompanied by TBARS increased values, however this was not observed on the present trial [75].

Indeed, we did not record a significant difference among treatments although it was observed an increased concentration of copper in liver in supplemented animals (Table 2), inferring those doses administered did not trigger a toxic effect. Similar results were obtained in Nellore steers supplemented with two levels and two copper sources (organic and inorganic) on metabolism of lipids [76].

Additionally, the short experimental period may have not been long enough to cause toxic concentration of copper in the liver. The mean liver copper concentration in supplemented lambs ranged between 247.86 and 341.29 mg/kg DM across treatments, which was lower when compared to other studies that observed hemolytic crisis from copper toxicity in lambs [41].

In another study to determine the effects of different sources and levels of copper on SOD plasma levels, lipid peroxidation and copper status in lambs, it was observed a reduction in plasma and hepatic concentrations of (MDA) the main marker for oxidative stress [54].

Copper was revealed to have an antioxidant effect in vivo but a pro-oxidant impact in v*itro* and its accumulation in tissues may tolerate oxidative stress [77]. This was not observed on the present trial as TBARS values in livers were not affected by treatments.

Homeostatic mechanisms maintain these functions by regulating physical properties of membranes, and how cells disrupt lipid homeostasis to bring about regulated cell death, presently possible to study through lipidomics analysis [78].

It is well known that the homeostasis process immediately after slaughter and a good welfare resting time of 4 to 8 hours before slaughter is effective in re-establishing homeostasis after transport to the abattoir, providing a greater length of the pre-rigor mortis period [79]. This may explain the absence of TBARS differences between treatments in liver, despite increase of SOD and GPx in this organ.

A comprehensive assessment of dietary intake and tolerable upper intake levels of any chemical element is needed to evaluate the long-term risk for public health and food risk assessment [80].

Table 5. TBARS averages, in mg/kg DM liver and LT muscle in Merino x Texel lambs receiving control diet or supplemented with different sources and levels of copper

TBARS (mg/kg)	Treatments (mg Cu /kg DM)					SEM
	Co	CuSO4	CuSO4	Cu-met	Cu-met	
	0	10	30	10	30	
Liver	0,899	0,988	1,02	0,515	0,974	0,046
LT muscle boning	0,164^{A}	0,146^{A}	0,172^{A}	0,079^{B}	0,105^{B}	0,026

Co = Control diet (no supplemented copper).
SEM= standard error of the mean.
Significant differences are denoted with different superscripts (P<0.05).

Although it was documented in this trial a significant effect of treatments (P<0.10) on TBARS values in LT muscle collected at boning (Table 5), supplemented animals showed lower values of TBARS when compared to control. Contrast analysis revealed a significant increase (P<0.05) on TBARS values in LT muscle from lambs receiving copper sulphate when compared to those fed Cu-methionine. The increase in antioxidant enzymes SOD and GPx in liver may explain the reduction in TBARS levels.

Other authors [54,81] did not find any effect of copper on the oxidative stability of LT muscle in lambs and goats collected at boning stage. Although lipid metabolism was affected by copper, the marbling scores and quality grades were not presently determined but considered similar in this other trial.

Selenium and vitamin E, powerful antioxidants, are essential micronutrients in sheep diets, both vital for growth and immune function, although the effect of vitamin E on post natal lamb survival is ambiguous [82].

Several authors claim that the combination of copper with vitamin E may give better results in meat stability and overall lamb carcass traits [83,84,85].

TBARS values of LT muscle immediately post vacuum freezing for 12 months did not differ significantly from those found at boning (Table 6) suggesting that lamb meat may be preserved frozen for at least 12 months, which complies for example with USA (FDA) requirements [86]. This may probably be justified by lack of oxygen in samples kept in vacuum and it can be deduced that there was a protective copper effect in maintaining stable a possible lipid oxidation during this period [87].

Our results are in agreement with previous data where the effects of chilled, frozen and chilled-then-frozen storage of lamb at various durations were evaluated on the quality and freshness of lamb [88].

Evidence of lipid oxidation was not found in any frozen lamb samples with copper supplementation and similar data was reported for frozen meat goat kids [89].

It should be highlighted the relationship between TBARS levels with rancid flavour. TBARS lower values than 0.56 mg/kg meat, obtained on the basis of an established methodology for determining oxidative rancidity [90] were considered acceptable [91] and non-contributing to rancid taste and odour factors also impacting the nutritive value [92].

Oxidative deterioration of lipids occurs at a faster rate at a reduced water content, and can occur via autoxidation or, less important, enzymatic oxidation. Hydrolytic rancidity describes the off flavours and aromas caused by release of short chain fatty acids from acylglycerols [93].

From the present trial it can be concluded that lamb meat obtained at slaughter and at 12 months post freezing did not manifest rancidity as the TBARS values were inferior to the cited threshold below 0.56 mg/kg meat. TBARS values recorded between lambs fed $CuSO_4$ and Cu-methionine (Table 5) were considered as low as not to be able to cause rancidity.

Concerning the lipid oxidative stability in LT muscle kept in a refrigerated exhibitor counter after 12 months freezing, TBARS values showed no significant effect among treatments on shelf life and display life, although a trend for linear growth of these values during the evaluation period. TBARS values on display life in LT muscle after 12 months vacuum freezing can be seen in Table 6.

Generally, the use of antioxidant supplements should be limited only to the cases in which oxidative stress is well documented therefore, even before any supplementation, it is necessary to measure oxidative stress to identify and eliminate the possible sources of free radicals and thus increased oxidative stress [97]. It should be enhanced that high cooper contents (e.g. 47 mg/kg diet) was previously tested in lambs for 60 days [94] with no detrimental effects.

In another trial the Cu status of mature, crossbred ewes, fed two sources (Cu sulphate vs Cu proteinate) and three levels (10, 20, or 30 mg/kg of dietary Cu) was determined in a 73 day feeding trial where the basal diet contained 5mg/kg copper. Feeding up to 30 mg/kg Cu from these sources did not cause an observable Cu toxicity during the 73 day period [95].

In the present trial and the previous one [41] it was found that copper supplementation in diets of Merino x Texel lambs, even at considered toxic levels of 39 mg/kg, resulted in increased hepatic concentration of antioxidant enzymes and did not influence copper levels in muscle oxidative stability.

These results may seem ambiguous, even misleading, as they were found to be non-toxic, neutral, and even beneficial on liver and muscle parameters.

Table 6. TBARS averages, in mg/kg DM LT muscle in Merino x Texel lambs Regardless of the source and dose of copper at Slaughter, immediately post vacuum freezing for 12 months and on shelf life and display life after 12 months freezing

Display Life	Time (days)				
	Slaughter	12 months	3 days defrost	6 days defrost	Time effect
TBARS (mg/kg)	$0,133 \pm 0,160^{C}$	$0,152 \pm 0,158^{C}$	$2,578 \pm 0,158^{B}$	$3,987 \pm 0,158^{A}$	Q<0,001

4. Concluding Remarks

Supplementation with copper without evidence of deficiency is not justified. No liver damage was observed, a parameter usually selected as the most reliable indicator of a long-term chronic ingestion of copper this trace element contributing significantly for the increase in the antioxidant enzymes SOD and GPx in liver.

Copper improved oxidative stability in muscle at boning and post 12 months vacuum freezing, demonstrating the antioxidant effect of this mineral. Furthermore, there was no pro-oxidant activity in liver indicating that basal diet with 9 mg copper/ kg DM, supplemented with 10 or 30 mg/kg DM copper sulphate or copper-methionine showed no adverse effects in liver or in muscle.

Present data, recorded on a significant (120 days) but not full part of the lifespan of lambs, contradict the well-established maximum risk levels in sheep (15-17 mg/kg DM) entrenched by an algorithm calculation, supporting the requirement for further trials.

5. Recommendations

Further studies are needed to assess effects associated with packaging and antioxidants on the quality of lamb and should combine copper, zinc with vitamin E and sulphur. Present results need replication since the highest dose used was more than double the recommended maximum and did not expose detrimental effects. Future studies should also measure the level of the four serum enzymes that are generally used in assessing liver functions which are alanine and aspartate aminotransaminase (ALT, AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) as the most sensitive indicators of hepatocyte injury.

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Contributions

CG: main corresponding author conducted all trials; CY: Design of the experiment, sampling, laboratory and analysis; TF: drafted the final version and updated the article; LC: sampling, laboratory analysis; AN: Design of the experiment; VB: collected and improved references and figures; MZ: Designed the experiment, sampling, data analysis and drafting the manuscript.

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Disclosure of Potential Conflicts of Interest

All authors declare that there is no conflict of interest.

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