

Article

Copper Trafficking in Sheep: A Pilot Proteomic Analysis of Enterocytes and Hepatocytes Under Dietary Copper Supplementation

Huey Yi Loh *  and Terry E. Engle

Department of Animal Sciences, Colorado State University, Fort Collins, CO 80523, USA;
terry.engle@colostate.edu

* Correspondence: huey.loh@colostate.edu

Simple Summary

Copper is an essential nutrient for sheep, due to its important roles in growth, energy production, nervous system function, and immunity. However, both copper deficiency and toxicity in sheep are common worldwide, showcasing challenges in proper copper supplementation. One reason may be that we still do not fully understand how copper is absorbed, transported, and excreted in sheep, as most previous research has been done in other species like mice and humans. In this study, sheep that received more copper had greater copper stored in the body, but the proteins responsible for transporting copper in the intestine and liver did not change. This suggests that sheep may lack the ability to effectively regulate how much copper they absorb, even when their diet contains more copper. We also found that copper concentration in bile and proteins involved in copper handling and excretion did not change with increased copper intake. This pilot study provided an explanation for why sheep are not efficient at regulating copper absorption and removing excess copper, which increased their risk of toxicity. Overall, these findings help provide insight into the mechanism of copper trafficking in sheep. More research is needed to better understand copper handling, especially at higher supplementation levels and across different sheep breeds.

Abstract

Seven Rambouillet-crossbred wethers were utilized to examine the impact of copper (Cu) supplementation on plasma, bile, liver, duodenum, jejunum, and ileum tissue Cu concentrations and Cu trafficking proteins abundance in the duodenum and liver. Wethers were assigned to one of two treatments: (1) control: no supplemental Cu and (2) +Cu: 12 mg of supplemental Cu/kg DM. After receiving treatments for 29 days, all wethers were harvested, and blood, bile, liver, duodenum, jejunum, and ileum samples were collected for Cu concentration analysis. Additional duodenum and liver samples were collected for proteomic analysis. Animals in the +Cu group had a greater ($p < 0.02$) Cu concentration in plasma, liver, and jejunum, but a lesser ($p < 0.01$) ileum Cu Concentration compared to controls. Duodenum and bile Cu concentrations and protein abundance for Cu trafficking proteins in the duodenum and liver were similar ($p > 0.10$) across treatments. In the +Cu group, 23 and 5 proteins were unique to the duodenum and liver, respectively, while the control group had 20 and 28 unique proteins in those tissues. These data indicate that known proteins responsible for Cu trafficking were not changed by the dose of Cu supplementation, ultimately allowing Cu to accumulate in the liver.



Academic Editors: Neila
Lidiany Ribeiro and Bing Wang

Received: 18 March 2026

Revised: 6 May 2026

Accepted: 7 May 2026

Published: 9 May 2026

Copyright: © 2026 by the authors.
Licensee MDPI, Basel, Switzerland.
This article is an open access article
distributed under the terms and
conditions of the [Creative Commons
Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

Keywords: deficiency; duodenum; homeostasis; liver; ruminants; toxicity

1. Introduction

Copper (Cu) is an essential trace element required by all living organisms for proper growth and development [1]. Copper serves as a cofactor for enzymes involved in a variety of biological functions such as respiration, cellular energy generation, immunity, free radical eradication, cross-linking of connective tissue, iron metabolism, and neurological function [2]. Due to the crucial role of Cu in biological processes, Cu deficiency can lead to hepatic, neurological, or immunological disorders [3,4]. In contrast, overconsumption of Cu can lead to liver necrosis, kidney failure, and eventually to death if not treated properly [5,6]. Ruminants, especially sheep, are susceptible to Cu toxicity as they have difficulty regulating the absorption and excretion of excess Cu, which makes Cu supplementation to ruminants extremely challenging [7–9]. The incidence of Cu deficiency and toxicity in sheep has been documented worldwide and has negatively impacted the economy of sheep-rearing countries, such as New Zealand, the United States, Australia, the United Kingdom, and many more [4,10].

One of the challenges of Cu supplementation to sheep arises from our lack of understanding of how Cu is being digested, absorbed, stored, metabolized, and excreted in sheep [11]. Currently, limited data are available on Cu homeostasis in ruminants (especially sheep), and most of the mechanisms associated with Cu homeostasis have been mapped by using humans [12], mice [13], and yeast [14] models. Although the process of intracellular Cu transport between species is expected to be highly conserved due to shared evolution of eukaryotic species, the difference in sensitivity to Cu toxicity between ruminants and humans may suggest differences in Cu homeostatic mechanisms. While Cu toxicity in sheep has been well documented [4,7], limited research has been conducted investigating the direct impact of Cu supplementation on the abundance of various Cu transport proteins within hepatocytes and enterocytes of sheep. Studies conducted by Simpson and colleagues [15,16] investigated copper trafficking proteins in North Ronaldsay and Cambridge sheep by analyzing protein abundance in the liver. However, no research has been conducted on the copper trafficking proteins abundance in the duodenum in sheep, and no data is available in both the liver and duodenum for the Rambouillet breed. It is well documented that there is a difference between breeds in copper tolerance [15,17,18], so the investigation of copper trafficking in Rambouillet sheep is essential to contribute to a better understanding of copper toxicity in this breed. Therefore, this pilot study aimed to investigate how Cu is being trafficked in ruminants using the Rambouillet sheep model. We hypothesized that the Cu concentration and Cu trafficking protein abundance in the duodenal and liver tissue of Rambouillet sheep receiving Cu supplementation would be greater than in sheep that did not receive additional Cu supplementation. Therefore, the objective of this pilot study was to examine the impact of Cu supplementation in sheep on plasma, bile, liver, duodenum, jejunum, and ileum tissue Cu concentrations and the differential abundance of Cu transport-related proteins in the duodenum and liver.

2. Materials and Methods

2.1. Ethical Statement

Prior to the initiation of this experiment, all animal care, handling, and procedures described herein were approved by the Colorado State University Animal Care and Use Committee (IACUC approval # 4293).

2.2. Animals and Diet

Eight Rambouillet crossed male sheep (body weight, BW = 45 ± 15.5 kg, 4–7 months of age) were selected and group-housed in a pen embedded with grass hay for diet adaptation. The basal diet (Table 1) was formulated based on the Nutrient Requirements of Small Ruminants [19] to meet the requirements for growing sheep. The sheep were fed once in the morning and had ad libitum access to feed and water. After adapting to the new environment, the sheep were surgically castrated 37 days prior to the initiation of the experiment. Blood collection via the jugular vein and liver biopsy were also conducted at this time to evaluate the initial Cu status of all animals. Blood was centrifuged at $2000 \times g$ to obtain plasma. Plasma and liver samples were analyzed for initial Cu concentrations. One sheep died prior to treatment initiation. Therefore, only seven animals were included in the study.

Table 1. Ingredient and nutrient composition of the basal diet.

Item	
Ingredient, % dry matter basis	
Cracked corn	30.0
Grass hay	33.3
Alfalfa hay	33.3
Soybean meal	3.4
Analyzed nutrient composition (Dry Matter; DM basis)	
DM, % as fed	90.9
Crude protein	12.7
Acid Detergent fiber, %	31.3
Neutral detergent fiber, %	47.1
Total digestible nutrient, %	67.0
NEm, Mcal/kg ¹	1.52
NEg, Mcal/kg ²	0.92
Calcium, %	0.69
Phosphorus, %	0.26
Magnesium, %	0.19
Potassium, %	1.84
Sodium, %	0.068
Sulfur, %	0.29
Iron, mg/kg	159
Zinc, mg/kg	21
Manganese, mg/kg	44
Copper, mg/kg	6.0
Molybdenum, mg/kg	2.0

¹ NEm = Net energy for maintenance. ² NEg = Net energy for gain.

Seven castrated sheep (hereafter, wethers; $n = 7$, BW = 56.5 ± 20 kg) were moved into individual pens (1.0 m $\times$ 2.5 m pens bedded with wood shavings and equipped with automatic waterers and individual feeders) 2 days prior to receiving dietary Cu treatments for adaptation to their new environment. The wethers were stratified based on age, BW, and initial liver Cu concentration into four blocks and randomly assigned to one of two treatments. Treatments consisted of: (1) control: no supplemental Cu ($n = 3$) and (2) +Cu: 12 mg of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /kg dry matter (DM; $n = 4$). Ground corn was used as the carrier to provide treatments. Treatment supplement for the +Cu group was prepared by thoroughly mixing $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ with ground corn, whereas the controls received only ground corn without $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ addition. The ground corn supplement was calculated as 1.1% of the total diet and fed to each animal separately before feeding the basal diet. This ensured the animals consumed all of their

supplement daily. The basal diet was composed of cracked corn, grass hay, alfalfa hay, and soybean meal, formulated to meet the requirements of finishing sheep according to NASEM [19] requirements. Daily intake was recorded on DM basis, and the feed amount was adjusted according to the animals' intake the day before throughout the experiment to ensure complete consumption of the basal diet. Daily dry matter intake for each animal was calculated using the total feed provided, subtracting the feed that remained the next day. The analyzed Cu concentration in the ground corn supplement in the control group was 2 mg Cu/kg DM, while the Cu concentration in the +Cu group was 500 mg Cu/kg DM. The basal diet contained 6 mg Cu/kg DM. Therefore, the average daily Cu intake of the control group throughout the experiment was 6.05 mg Cu/kg DM (6 mg/kg from diet + 0.05 mg/kg from supplement), whereas the +Cu group received a total of 19.7 mg Cu/kg DM (6 mg/kg from diet + 13.7 mg/kg from supplement).

Wethers received the dietary treatment for 29 days before slaughter. On day 30 of the experiment, all wethers were transported to a USDA-inspected abattoir and harvested. Blood, bile, liver, and duodenum, jejunum, and ileum samples were collected at the time of slaughter. Blood was collected into trace mineral-free blood collection tubes containing 10.8 mg K₂EDTA (BD 368381) and centrifuged at 2000× *g* to obtain plasma. Approximately 50 g (wet weight) of liver, duodenum, jejunum, and ileum samples were washed with phosphate-buffered saline (PBS) 3 times before transferring to storage tubes. All samples were stored at room temperature during the collection period and immediately transferred to −20 °C for storage until Cu analysis was performed. Additional liver and duodenum samples (approximately 20 g; wet weight) were collected for proteomic analysis. Liver and duodenum samples for proteomic analysis were washed 3 times with PBS and placed on ice until transferred to a −80 °C freezer. Enterocytes from the duodenum tissues were separated from the serosa by scraping using a scalpel blade as described by Han et al. [20] before storing at −80 °C. All samples were collected within an hour after stunning to minimize protein degradation.

2.3. Copper Analysis

All samples were processed and analyzed for Cu concentrations following procedures described by Loh et al. [21]. Briefly, approximately 1 g of tissue samples (liver, duodenum, jejunum, and ileum) were dried for 48 h to obtain the DM weight and ashed for 24 h to obtain ash, then resuspended in a solution with 1.2 M hydrochloric acid. Samples were brought up to 5 mL total volume with deionized water for mineral analysis. For liquid samples, one mL of the sample was added to a 15 mL conical tube, followed by another one mL of 70% nitric acid. The tubes were left under a fume hood for 10 min before transferring to a 70 °C water bath for 4 h. After 4 h, the tubes were removed from the water bath and chilled to room temperature. One mL of 30% hydrogen peroxide was added to each tube, and the tubes were placed back in the 70 °C water bath for another 2 h. After incubation, each tube was brought to a total volume of 5.0 mL with ultrapure water. Copper concentration was analyzed using atomic absorption spectroscopy (PinAAcle 500, PerkinElmer, Waltham, MA, USA).

2.4. Proteomic Analysis

2.4.1. Protein and Peptides Extraction

Protein extraction and peptide preparation for proteomic analysis were conducted using Pierce BCA Protein Assay Kit and EasyPep Mini MS Sample Prep Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 100 mg of tissue was weighed into a bead-beating tube containing 1 mL Thermo Lysis Buffer, 20 µg of 0.1 mm zirconia silica beads, 3 µL Universal Nuclease, and 7.5 µL HALT

Protease Inhibitor Cocktail. Tubes were subjected to 5 cycles of bead beating for 30 s at 6 m/s at room temperature using a bead mill homogenizer (Fisherbrand™ Bead Mill 24 Homogenizer, Thermo Fisher Scientific, Waltham, MA, USA), with 2 min dwell time on ice between cycles to avoid sample overheating. The supernatant layer was transferred to a new tube after being centrifuged at $8000\times g$ for 5 min. Protein concentration was quantified using a BioRad 680 microplate reader (BioRad Laboratories, Hercules, CA, USA) with the software Microplate Manager (BioRad, Version 5.2 Build 103, Hercules, CA, USA), and 100 µg of total protein was aliquoted from each sample. The protein aliquot was raised to 100 µL with lysis buffer, followed by the addition of reduction and alkylation solutions sequentially. The solution was incubated at 95 °C in a dry block heater (VWR International, Radnor, PA, USA) for 10 min. After cooling to room temperature, 50 µL of a 0.2 µg/µL Trypsin/LysC mixture was added, and samples were digested with shaking using a shaker device at speed 2 (Ohaus G560 Vortex-Genie® 2, Scientific Industries Inc., Bohemia, NY, USA) at 37 °C for 2 h. Digestion Stop Solution was added to deactivate the enzymes, then, pre-formulated buffers and a mixed-mode resin to get rid of the hydrophilic and hydrophobic contaminants were used for peptide clean up (EasyPep™ MS Sample Prep Kit A4006, Thermo Fisher Scientific, Waltham, MA, USA). Peptide eluate was dried in a vacuum evaporator (Thermo Savant AES2010-220 Centrifugal Evaporator, Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in 3% acetonitrile/0.1% formic acid and quantified using NanoDrop at 205 nm (NanoDrop One/OneC Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) [22].

2.4.2. Mass Spectrometry Analysis—LC-MS/MS

Reverse-phase chromatography was performed using water with 0.1% formic acid (A) and 80% acetonitrile with 0.1% formic acid (B). One µg of peptides was purified and concentrated using an online trap column (PepMap Neo C18 5 µm, 300 µm ID $\times$ 0.5 cm, Thermo Fisher Scientific, Waltham, MA, USA). Subsequent chromatographic separation was performed on a Vanquish Neo (Thermo Fisher Scientific, Waltham, MA, USA) on a reverse phase nanospray column with integrated silica emitter (Ionopticks Aurora Ultimate Gen 3 C18 1.7 µm, 75 µm ID $\times$ 25 cm column, 45 °C) using a 90 min gradient at a flow rate of 300 nanoliters/min: 1–6% B over 3 min followed by 6–35% B over 70 min, 35–45% B over 5 min ending in 12 min of washing at 500 nanoliters/minute, 99% B. Peptides were eluted directly into the mass spectrometer (Orbitrap Eclipse, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Nanospray Flex ion source and spectra were collected over a m/z range of 375–2000 under positive mode ionization. Ions with charge state +2 or higher were accepted for MS/MS using a dynamic exclusion limit of 1 MS/MS spectra of a given m/z value with an exclusion duration of 60 s. The instrument was operated in Fourier-transform mode for MS detection (profile; resolution of 240,000) and ion trap mode for MS/MS detection with a normalized high-energy C-trap dissociation collision energy set to 30% and data centroided.

2.4.3. Data Processing

Data processing was conducted on Proteome Discoverer 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Additional precursors within the isolation window of the precursor spectrum, when chimeric or mixed spectra are present, were identified by the precursor detector node with S/N = 1.5. Spectra from all samples were searched using the Sequest HT [23] node, setting methionine oxidation and protein N-7 terminus acetylation as dynamic and cysteine Carbamidomethylation as fixed modification. Raw data was interrogated against the FASTA file of the reference proteome of *Ovis aries* (taxon ID 9940) from UniProt [24]. Additionally, the cRAP proteome (The common Repository of Adven-

titious Proteins -cRAP- contains commonly found contaminant proteins in proteomics experiments; cRAP_100518) was included. Sequest HT was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10 ppm. Peptide spectral matches (PSMs) were validated using the Percolator node [25]. Thresholds were set such that a false discovery rate (FDR) of $\leq 1\%$ and protein identification was defined with at least one unique peptide.

2.5. Statistical Analysis

All statistical analyses were conducted using R statistical software version 4.3.2 [26] in RStudio version 2024.04.2+764. Power analysis was conducted prior to the initiation of the experiment using hepatic Cu concentration and proteomic data. An alpha of 0.05, 2 treatment groups, and $n = 4$ were applied for both variables. Power values of 0.86 and 0.90 were achieved for proteomic and hepatic Cu outcomes, respectively, assuming group differences of 1 and 50 and standard deviations of 0.3 and 13. A linear mixed model was fitted to investigate the effect of Cu supplementation on Cu concentration in plasma, liver, bile, and small intestine tissues, with initial Cu or BW concentration as the covariate and block as the blocking factor. An individual animal was used as the experimental unit. Statistical significance was determined at $p \leq 0.05$.

Total peptide abundance values were normalized before analysis by dividing the abundances by the normalization factor. The normalization factor was the factor of the sum of the sample and the maximum sum in all files. All 7 samples were included in the analysis, and only proteins with quantification values in at least 2 replicates (2/3) and three (3/4) replicates in control and +Cu, respectively, were included. The protein intensity value was transformed into Log2, and pairwise analysis was conducted. Log2 fold change was calculated, and Benjamini–Hochberg (BH) adjustment was applied. Statistical significance was defined by a BH-corrected p -value ≤ 0.05 and a Log2 fold change in either ≤ -1 or ≥ 1 . Proteins that have quantitative values in at least 2 out of 3 total replicates (control) or 3 out of 4 total replicates (+Cu), but no values in any replicates of another treatment were identified.

3. Results

3.1. Animal Performance and Copper Concentration

Average dry matter intake (DMI), initial and final BW, liver and plasma, duodenum, jejunum, ileum, and bile Cu concentrations are summarized in Table 2. At the end of the experiment, +Cu sheep had greater ($p < 0.05$) final plasma, final liver, and jejunum Cu concentrations compared to the controls. Final body weight and Cu concentrations in ileum tissues were greater ($p < 0.01$) in the controls compared to the +Cu group. Initial BW, DMI, and Cu concentrations in initial plasma, initial liver, duodenum, and bile were similar ($p > 0.10$) between treatment groups.

Table 2. Influence of copper supplementation for 29 days on body weight and copper concentration in liver, plasma, duodenum, jejunum, ileum, and bile in wethers.

Item	Treatment ¹		SEM ²	<i>p</i> -Value
	Control	+Cu		
<i>n</i> =	3	4		
Initial body weight, kg	57.33	55.88	4.12	0.20
Final body weight, kg	58.48	55.45	3.69	<0.01
Dry matter intake, kg/d	1.88	1.69	0.12	0.49
Initial liver Cu concentration, mg/kg	87.50	94.94	4.65	0.48

Table 2. Cont.

Item	Treatment ¹		SEM ²	p-Value
	Control	+Cu		
Final liver Cu concentration, mg/kg	94.54	199.58	38.83	0.04
Initial plasma Cu concentration, mg/L	0.93	0.80	0.10	0.53
Final plasma Cu concentration, mg/L	0.90	1.01	0.03	<0.01
Duodenum Cu Concentration, mg/kg	6.87	14.10	3.46	0.14
Jejunum Cu Concentration, mg/kg	14.76	14.84	6.03	<0.01
Ileum Cu Concentration, mg/kg	16.36	10.20	3.23	<0.01
Bile Cu Concentration, mg/L	0.88	0.75	0.15	0.67

¹ Treatment = Control: sheep received no supplemental Cu for 29 days. +Cu: sheep received 12 mg of supplemental Cu from CuSO₄·5H₂O/kg DM for 29 days. ² SEM = Standard error of the means.

3.2. Proteomic Analysis

Pre-normalization and normalized abundance of protein were highly consistent across all samples for both liver and duodenum samples (Supplemental Figures S1–S3). A total of 5580 proteins and 52,956 peptides were identified in the liver samples, and 99.55% of the identified proteins were from the sheep proteome. A total of 7304 proteins and 72,076 peptides were identified in the duodenum samples, and 99.78% of those were from the sheep proteome. The contaminant proteins (0.45% and 0.22%) identified were the ones commonly observed in proteomics analysis and were removed from the study.

There was no difference ($p > 0.10$) in protein abundance between the treatments for all proteins in the liver and duodenum. The comparison of Cu trafficking proteins in the liver and duodenum between treatment groups is summarized in Tables 3 and 4, respectively.

Table 3. Log₂ fold change in selected copper trafficking related proteins in the liver tissue of control sheep (no supplemental Cu) and +Cu sheep (sheep supplemented with 12 mg of Cu from CuSO₄·5H₂O/kg DM) for 29 days.

Protein Description	Accession Number ¹	Log 2 Fold Change ²	Adjusted p-Value
Copper homeostasis protein cutC homolog	W5PTA5	0.41	0.99
Copper transport protein	A0A836D4R3	0.05	0.99
Cytochrome C oxidase Copper Chaperone	A0A6P3DZL9	−0.12	0.99
Glutathione synthetase	W5PMH1	−0.11	0.99
Metallothionein-1B	P09577	−0.01	1.00
NADH-cytochrome b5 reductase	A0A835ZX95	−0.53	0.99
P-type Cu(+) transporter	W5PH10	0.31	0.99
Superoxide dismutase [Cu-Zn]	P09670	0.28	0.99
Superoxide dismutase [Cu-Zn]	A0A6P3THN6	−0.39	0.99
Superoxide dismutase [Cu-Zn]	B9VGZ6	0.11	0.99
Superoxide dismutase copper chaperone	A0A835ZMZ6	0.08	0.99

¹ Accession number = Uniprot accession number. ² Log₂ fold change = +Cu/Control purge group; positive number = Greater in +Cu vs. Control purge group; negative number = Greater in Control vs. +Cu group. Control: No supplemental Cu and +Cu: 12 mg of supplemental Cu from CuSO₄·5H₂O/kg DM.

Proteins that were exclusively present in the liver tissues of the control and treatment groups are presented in Tables 5 and 6, respectively. There were five and 28 proteins exclusively present in the control and +Cu group, respectively. Tables 7 and 8 summarize proteins that are exclusively present in the duodenum in the control and +Cu groups, respectively. Twenty-three proteins were found to be exclusively present in the duodenum of the control group, while 20 proteins were exclusively present in the duodenum of the +Cu group. Proteins that were labeled as “Uncharacterized Protein” were renamed using BLAST in UniProt [27], and the protein with the lowest expectation value was selected.

Table 4. Log₂ fold change in selected copper trafficking related proteins in the duodenum tissue of control sheep (no supplemental Cu) and +Cu sheep (sheep supplemented with 12 mg of Cu from CuSO₄·5H₂O/kg DM) for 29 days.

Protein Description	Accession Number ¹	Log 2 Fold Change ²	Adjusted <i>p</i> -Value
Copper homeostasis protein cutC homolog	W5PTA5	0.52	0.97
Copper transport protein	A0A836D4R3	−0.09	0.97
Cytochrome C oxidase Copper Chaperone	A0A6P3DZL9	−0.06	1.00
Glutathione synthetase	A0A836A7V3	−0.04	0.99
Metallothionein-1B	P09577	0.50	0.97
NADH-cytochrome b5 reductase	A0A6P3YIV0	0.06	0.97
P-type Cu(+) transporter	A0A835ZFY2	0.06	1.00
Superoxide dismutase [Cu-Zn]	P09670	0.29	0.97
Superoxide dismutase [Cu-Zn]	A0A6P3THN6	−0.09	0.99
Superoxide dismutase copper chaperone	W5PBW4	0.60	0.97

¹ Accession number = Uniprot accession number. ² Log₂ fold change = +Cu/Control purge group; positive number = Greater in +Cu vs. Control purge group; negative number = Greater in Control vs. +Cu group; Control: No supplemental Cu and +Cu: 12 mg of supplemental Cu from CuSO₄·5H₂O/kg DM.

Table 5. Proteins that were exclusively present in the liver tissue of control sheep ¹.

Protein Description	Accession Number ²	Subcellular Location
3'-phosphoadenosine 5'-phosphosulfate synthase 1	W5PG10	Cytoplasm
Proteasome maturation protein	W5PRA5	Cytoplasm/Nucleus
Keratin 83	E3VW85	Extracellular space
Golgi SNAP receptor complex member 1	W5PMP6	Golgi apparatus
Fibrillar collagen NC1 domain-containing protein	A0A836A7X7	Secreted

¹ Control sheep: Sheep that received only a basal diet and no supplemental copper for 29 days. ² Accession number = Uniprot accession number.

Table 6. Proteins that were exclusively present in the liver tissue of the +Cu sheep ¹.

Protein Description	Accession Number ²	Subcellular Location
Cilia- and flagella-associated protein 69	A0A836AIA2	Cytoplasm
Dipeptidyl peptidase 8	W5Q971	Cytoplasm
Dynein light chain Tctex-type 1	A0A6P7ECY5	Cytoplasm
Protein-serine/threonine phosphatase	A0A836CRW4	Cytoplasm
Transportin 2	W5PQ43	Cytoplasm/Nucleus
F-box domain-containing protein	A0A6P7DSW6	Cytoplasm/Nucleus
Formin-binding protein 1-like	W5Q5W6	Cytoplasm/Plasma membrane
GTPase IMAP family member 4-like	W5NVR0	Cytosol
Dihydrodiol dehydrogenase	W5PSA0	Cytosol/Mitochondria
Trafficking protein particle complex 8	W5P8B4	Endoplasmic reticulum/Golgi apparatus
Glutamyl-peptide cyclotransferase	W5PKH9	Golgi apparatus
Polypeptide N-acetylgalactosaminyltransferase	A0A836AL93	Golgi apparatus
Trafficking protein particle complex 6A ³	A0A6P7ET14	Golgi apparatus
Hemoglobin subunit beta (Fragment)	A0A7S9SST0	Heptoglobin-hemoglobin complex
Sphingolipid transporter 1 (putative)	W5NTF8	Lysosome
Vacuolar protein sorting-associated protein 11 homolog	W5PTD1	Lysosome

Table 6. Cont.

Protein Description	Accession Number ²	Subcellular Location
Ig-like domain-containing protein	A0A836CSA5	Membrane
Isocitrate dehydrogenase [NAD] subunit, mitochondrial	W5P8N8	Mitochondrion
Isocitrate dehydrogenase [NAD] subunit, mitochondrial	A0A6P7DMM7	Mitochondrion
LYR motif-containing protein 2	A0A6P3EGX4	Mitochondrion
F-box and leucine-rich repeat protein 20	W5PMA6	Nucleus
O-acyl-ADP-ribose deacylase 1	A0A6P3T7T7	Nucleus
Cleavage and polyadenylation specificity factor subunit 1	A0A836AB03	Nucleus
Ig-like domain-containing protein	W5PSC1	Plasma membrane
MHC class II DQA	W1I769	Plasma membrane
Ninjurin-1	A0A836AC10	Plasma membrane
Vesicle transport protein	A0A6P3YN43	Plasma membrane
Von Willebrand factor	A0A836D5W7	Secreted

¹ +Cu sheep: Sheep that received only a basal diet + 12 mg of supplemental Cu from CuSO₄·5H₂O/kg DM for 29 days. ² Accession number = Uniprot accession number. ³ These proteins were initially labeled “Uncharacterized”. The FASTA sequence of the protein was used to match each protein to a more commonly recognized or accepted name using the UniProt BLAST feature.

Table 7. Proteins that were exclusively present in the duodenum of the control sheep ¹.

Protein Description	Accession Number ²	Subcellular Location
Peptidase S1 domain-containing protein	W5NYB0	Cytoplasm
Rab-GAP TBC domain-containing protein	A0A836A5V5	Cytoplasm
Chloride intracellular channel protein ³	A0A836CTI0	Cytoplasm
2'-deoxynucleoside 5'-phosphate N-hydrolase 1	A0A836CTH1	Cytoplasm/Nucleus
Condensin complex subunit 2	W5PWU1	Cytoplasm/Nucleus
Inhibitor of nuclear factor kappa-B kinase subunit alpha	A0A835ZPV0	Cytoplasm/Nucleus
Acrosin-binding protein	A0A836D5L5	Cytoplasmic vesicle/Secretory vesicles
UDP-glucuronosyltransferase	A0A836D2A1	Endoplasmic reticulum
Vitamin-K-epoxide reductase (warfarin-sensitive)	W5PFE3	Endoplasmic reticulum
Collectin-46	W5NXU4	Extracellular space
Eukaryotic elongation factor, selenocysteine-tRNA specific	W5P3C2	Mitochondrion
Pentatricopeptide repeat domain 3	W5QHG2	Mitochondrion
Probable glutamate--trna ligase, mitochondrial	W5Q6N0	Mitochondrion
General transcription and DNA repair factor IIH subunit TFB5	A0A836AC27	Nucleus
Histone H2A	A0A836CTR7	Nucleus
Ras homolog family member F, filopodia associated	W5PTZ3	Nucleus
RING-type domain-containing protein	A0A836D5B6	Nucleus
SS18 N-terminal domain-containing protein	A0A836CSX3	Nucleus
Chorein N-terminal domain-containing protein	A0A836AKE8	Organelle contact sites
Protein-tyrosine-phosphatase	A0A6P7E3E7	Plasma membrane
Pleckstrin-2 ³	A0A6P3EGB6	Plasma membrane
39S ribosomal protein L34, mitochondrial	A0A836D2N2	Ribosome
TSC complex subunit 1	W5P6L8	TSC1-TSC2 complex

¹ Control sheep: Sheep that received only a basal diet and no supplemental copper for 29 days. ² Accession number = Uniprot accession number. ³ These proteins were initially labeled “Uncharacterized”. The FASTA sequence of the protein was used to match each protein to a more commonly recognized or accepted name using the UniProt BLAST feature.

Table 8. Proteins that were exclusively present in the duodenal tissues of +Cu sheep ¹.

Protein Description	Accession Number ²	Subcellular Location
Actin-binding LIM protein 1	A0A836CRD7	Cytoplasm
Peptidase S1 domain-containing protein	W5P415	Cytoplasm
Coiled-coil domain-containing protein 71L	A0A836ADZ9	Cytoplasm
C-type lectin domain-containing protein	W5QGF9	Extracellular space
Fucosyltransferase	A0A6P7EYT4	Golgi apparatus
Beta-1,4-galactosyltransferase	A0A836AGX1	Golgi apparatus
Beta-glucuronidase	A0A835ZMV0	Lysosome
Peptidyl-prolyl cis-trans isomerase E	W5QFV9	Mitochondrion
Zinc finger MYND domain-containing protein 11	A0A6P3YKT9	Nucleus
Histone deacetylase	W5PAD5	Nucleus
BTB domain-containing protein ³	A0A6P3YH93	Nucleus
ABC-type glutathione-S-conjugate transporter	A0A6P7EJI1	Plasma membrane
Sugar transporter SWEET	W5P0N7	Plasma membrane
ABC transporter domain-containing protein	A0A836CRS2	Plasma membrane
Ig-like domain-containing protein	A0A836CTX9	Plasma membrane
Diacylglycerol kinase	A0A6P7E7Q1	Plasma membrane
UBA domain-containing protein	W5Q307	Plasma membrane/Golgi apparatus
Carboxypeptidase A1	A0A836AH38	Secreted
Phospholipase A2	W5PR24	Secreted
Chromosome 14 C16orf70 homolog	W5NXG6	Trans-golgi network

¹ +Cu sheep: Sheep that received only a basal diet + 12 mg of supplemental Cu from CuSO₄·5H₂O/kg DM for 29 days. ² Accession number = Uniprot accession number. ³ These proteins were initially labeled “Uncharacterized”. The FASTA sequence of the protein was used to match each protein to a more commonly recognized or accepted name using the UniProt BLAST feature.

4. Discussion

Copper is an essential trace mineral that is widely supplemented to livestock to prevent Cu deficiency. The recommended copper supplementation levels according to NASEM [19] are 7–11 mg/kg DM. However, Cu imbalance remains a problem for sheep and cattle production, as Cu metabolism in ruminants is still poorly understood [11,28]. The fundamental knowledge of cellular Cu homeostasis has been produced with non-ruminant models, but a greater susceptibility to copper toxicity in ruminants compared to humans indicates that there is a difference in Cu metabolism and homeostasis between ruminants and humans [29]. The current understanding of Cu transport in ruminants is based on the assumption that Cu metabolism, at the cellular level across species, is consistent due to the shared evolution of eukaryotes [30]. For detailed assumptions of current Cu transport models within enterocytes and hepatocytes, see Clarkson et al. [11] and López-Alonso and Miranda [31].

Briefly, once in the intestinal lumen, Cu²⁺ can be reduced to Cu¹⁺ by a membrane-bound reductase enzyme (cytochrome B reductase 1; Cybrd1) and is transported by the Cu transporter protein (CTR1) into the enterocyte [32–34]. Copper transport within enterocytes is mediated by several proteins: glutathione (GSH), antioxidant 1 (ATOX1), Cyclo-oxygenase 17 (COX17), Cu chaperone for superoxide dismutase (CCS), and Menkes protein (ATP7A). Cu will immediately be incorporated into Cu cytochrome c oxidase (CCO) and superoxide dismutase (SOD) for cellular function [11,35,36]. The surplus of Cu will bind to metallothionein (Mt) and be held in the lysosome [37]. Once the amount of Cu in the cell exceeds the carrying capacity in the lysosome, excess Cu will be excreted into the blood via ATP7A [30]. Copper transport within the blood is chaperoned by albumin, transcuprein, and ceruloplasmin [38,39]. Absorbed Cu is transported to the liver, which plays a major role in Cu regulation and storage. Copper uptake, transport, and storage in

the hepatocytes are similar to those in enterocytes, with a few exceptions. Wilson disease protein (ATP7B) was found in hepatocytes and plays a role in incorporating Cu into ceruloplasmin to be returned into circulation for distribution, but ATP7A was not expressed in the liver [40]. Excess hepatic Cu is incorporated into the bile to be excreted by Cu metabolism via ATP7B, MURR1 domain (COMMD1), and potentially X-linked inhibitor of apoptosis protein (XIAP) [41–43].

To improve our understanding of Cu transport in sheep in comparison to the models established in other species, this study was conducted as an exploratory analysis to evaluate the similarity of copper trafficking mechanisms between sheep and other species. It is important to note that the extrapolation of the results should be done cautiously due to the small sample size used in this study. Despite the differences in dietary Cu concentration in the current study, CTR1 abundance in the duodenum remained similar across treatments, suggesting that Rambouillet sheep may lack the ability to regulate Cu absorption in the small intestine. Without regulation, more Cu will be absorbed into the blood when there is an increase in dietary Cu concentration, which was verified by the higher hepatic Cu concentration in the +Cu group. It is important to note that Cu could also be taken up into the enterocytes via other pathways (e.g., DMT1 [44] or anion transporter [45]), but CTR1 remains the primary route that is responsible for the majority of copper uptake [46]. Similar to CTR1, Cu transport proteins (Glutathione synthetase; GSS, CCS, CCO, SOD) within cells remained similar across treatments, but we failed to identify the presence of COX17 and ATOX1 in the cell, which are Cu chaperones that are present in enterocytes of other species [36,47]. These proteins (COX17 and ATOX1) were found in yeast models [47], but no work has been conducted in sheep duodenum to confirm the absence or presence of these proteins in the duodenum. In conjunction with similar Mt abundance within enterocytes, we did not observe changes in duodenum Cu concentration. We identified a P-type Cu⁺ transporter in the current study, but we were not able to differentiate between ATP7A and ATP7B, or whether this was a new form of transporter that was not characterized in the reference proteome. Analysis of RNA was conducted in soft-horned tissue of Tibetan sheep, and expression of the ATP7A gene was identified [48], but to our knowledge, no work has been conducted to investigate the presence of the P-type Cu⁺ transporter protein in sheep. In general, the P-type Cu⁺ transporter aids in moving Cu across cellular membranes [49]. With Cu concentration within the enterocytes exceeding the storage capacity, the P-type Cu⁺ transporter transports the excess Cu into the bloodstream from the enterocytes.

As there was no difference detected in liver CTR1 abundance between treatments, it appears that more Cu was being absorbed and stored in the liver in the +Cu group due to higher Cu concentration in the blood (from greater Cu absorption in the duodenum). The Cu concentration in bile did not increase with increasing dietary Cu in the current study. This finding aligned with a previous study conducted in Norwegian Dala breed sheep, which showed that Cu was not excreted in the bile after receiving 1.25mCi of ⁶⁴Cu intravenously [50]. In contrast, studies conducted by Deol et al. [51] and Minervino et al. [52] reported a higher Cu concentration in the bile with increased Cu intake in sheep. Deol et al. [51] supplemented 20 mg/kg DM of Cu to Merino sheep for 15 weeks, while Minervino et al. [52] supplemented 450 mg/kg DM to Santa Inês crossbred sheep for 14 weeks, and they observed an increase in Cu concentration in the bile of animals that received Cu supplementation. The differences in biliary excretion of Cu between studies may be due to breed differences and/or type of copper supplement, suggesting that further study is required to investigate the impact of breed and type of copper supplement on copper trafficking and excretion mechanisms. Despite the differences in biliary Cu concentrations reported in previous studies, the Cu concentration in bile remains very low relative to the amount ingested, indicating the inefficiency of Cu excretion via bile in sheep.

to prevent liver Cu accumulation [50–52]. This inefficiency in biliary Cu excretion may be due to the similar abundance of the P-type Cu⁺ transporter in the liver observed in this study. The P-type Cu⁺ transporter is believed to facilitate Cu incorporation into bile for excretion [53].

Copper toxicity can be fatal, as excess Cu can lead to the generation of reactive oxygen species (ROS) that can cause oxidative damage in the cell, ultimately leading to apoptosis [54]. Proteins that assist with oxidative stress mitigation and/or resistance, such as SOD, Mt, and GSS, were similar between treatments in the current experiment. This finding aligns with findings by Saylor and Leach [55], who reported that sheep cannot synthesize metallothionein in response to increased dietary Cu. While no differences in Cu trafficking protein abundance were detected across treatments in the current study, the potential regulation at other levels (e.g., phosphorylation or ubiquitination) or altered subcellular localization could be happening with different dietary Cu intake. As Cu homeostasis is known to be tightly controlled within a biological system, future studies incorporating targeted approaches to investigate these other potential regulations are warranted to better understand copper homeostasis in sheep with an ultimate goal of minimizing Cu toxicity incidents in sheep.

The accumulation of Cu in the liver of the +Cu group was expected, as the previous literature has documented the incremental increase in hepatic Cu concentration (58, 191, 297 mg Cu/kg of fresh liver) with each increase in dietary Cu concentrations (2.2, 11.3, 47.0 mg Cu/kg diet on DM basis) [55]. A study conducted by Simpson et al. [15] compared liver Cu concentration and hepatic protein abundance in sheep consuming a Cu-supplemented diet (155 mg Cu/kg DM) and sheep consuming a diet without supplementation (≤ 50 mg Cu/kg DM). They observed moderate Cu overload (~ 450 mg/kg DM) in the liver of supplemented sheep after consuming the diet for 3 months, but by 3.5 months, hepatic Cu concentration increased drastically to approximately 1000 mg/kg. The study also identified concentration differences in some Cu-responsive proteins (aldehyde dehydrogenase, apolipoprotein A-1, etc.) in the liver [15], but the proteomic analysis conducted in this study did not observe differences in the abundance of any Cu homeostatic proteins in the liver. The discrepancy between studies may be due to breed differences, where the current study utilized Rambouillet sheep, while the study conducted by Simpson et al. [15] utilized North Ronaldsay sheep. In addition, the length of the study (29 days), as determined by referencing the previous study [56] that demonstrated the alterations in protein abundance in the intestine of pigs after 28 days of dietary treatment, may not be enough to trigger the adaptation and change of copper proteins in the intestine and liver of the sheep. The discrepancy between studies could also be due to the differences in Cu supplementation concentrations, where the Cu supplementation dose used in the previous study by Simpson et al. [15] was 155 mg/kg diet DM compared to the much lower concentrations (12 mg Cu/kg diet DM) used in the current study. We chose these supplementation concentrations according to the NASEM [19] nutrient requirement to prevent potential Cu toxicity in sheep, but it might not have been enough to trigger the change in Cu trafficking protein concentration. Although we supplemented relatively low Cu concentrations to the sheep in the current study, the hepatic Cu concentration was above the value for liver Cu concentrations (approximately 83 Cu/kg dry weight) set by previous studies [57,58], but the animals did not show any clinical signs of Cu toxicity. Plasma Cu concentration in the +Cu group was greater compared to the control group in the current study. This is similar to the findings from Dezfoulan et al. [59], where sheep consuming 10 and 20 mg/kg of supplemental Cu had elevated plasma Cu concentration compared to the control group that did not receive any supplemental Cu (basal diet contained 8.6 mg Cu/kg DM). We observed a statistical difference in Cu concentration in the jejunum, but Cu concentration in

the jejunum of the +Cu group was only 0.08 mg/kg greater than that of the control group, so it may not be biologically significant. This study also observed a 6.16 mg/kg lower Cu concentration in the ileum in the +Cu group compared to the control. The findings in the jejunum and ileum suggest further protein abundance investigation in those sections of the gastrointestinal tract for future research.

In the current study, there were multiple proteins found to be “exclusively” present in both tissues in the control and +Cu group. However, there were no specific trends of increased or decreased abundance of specific biochemical processes for the function of the proteins extracted from UniProt [60]. If the information was not available on UniProt, the protein function was extracted from the previous literature (Supplementary Tables S1–S4). The proteins that were only present in the duodenum of the control animals are involved in catalysis, proteasomal degradation, lipid transfer [60], intracellular communication [61], immunity, transcription and translation regulation [60], aiding in the resistance of tumor cells to metabolic stress [62], and cell growth and size regulation [60]. Proteins that were only found in the duodenum of +Cu sheep are involved in energy metabolism, protein, peptide, and amino acids modification and degradation, the immune system, sugar transportation across membranes, autophagy, and transcription regulation [60]. Sugar transporter SWEET was only present in the +Cu group, which may signify the increase in glucose absorption in the small intestine. However, despite this potential enhancement in glucose uptake, the final BW of the +Cu group remained lower than that of the control group.

In the liver of the control group, the exclusive proteins identified are reported to be involved in esophageal squamous cell carcinoma suppression [63], sulfur metabolism, sulfation reaction, hair formation, and proteasome biogenesis. However, hepatic proteins that were present only in the +Cu group have been reported to be involved in energy metabolism, respiration, immune regulation and function, cellular transportation, protein–protein interaction, autophagy and secretory trafficking, protein degradation, modification, and glycosylation, assembly and facilitating of enzyme function, lung tumor formation, and development of Alzheimer’s disease. Copper, due to its ability to cause oxidative stress and cellular damage, is often linked to cell death and is used in potential cancer treatment. In our study, a protein that protects tumor cells from metabolic stress in the duodenum was identified in sheep that did not receive additional Cu supplementation. However, a different protein that aids in cell carcinoma suppression was identified in the liver of the same group. In addition, a protein that aids in lung tumor formation was identified in the liver of sheep that received additional dietary Cu. The reason behind the influence of Cu supplementation in sheep on proteins that are exclusively present in one treatment but not the other treatment is unknown. The current study serves as a pilot study to understand the Cu trafficking mechanism in the duodenum and liver of sheep. Due to the small sample size in the current study, the interpretation of the results is limited. Future research with more replicates is warranted to understand the impact of Cu supplementation on biological processes in sheep. In addition, due to potential breed differences and the exploratory nature of this study, extrapolation of these findings should be performed cautiously, and further research on the influence of breed on copper trafficking is warranted.

5. Conclusions

Copper imbalance in sheep remains a problem worldwide, and producers need to supplement Cu properly to prevent Cu deficiency and toxicity. The understanding of Cu homeostasis in ruminants has been based on non-ruminant models, but the high prevalence of Cu toxicity in ruminants suggests differences in Cu homeostasis between monogastric and ruminant animals. This study served as a preliminary investigation to understand proteins involved in Cu absorption, storage, and excretion in sheep. In general, this study

showed that sheep receiving approximately 12 mg/kg DM of Cu supplementation did not alter Cu absorption proteins in the small intestine to downregulate Cu absorption. Although there was an increase in hepatic Cu uptake, the proteins responsible for Cu excretion did not increase, which led to the accumulation of Cu in the liver. When hepatic Cu concentration increases, the liver of sheep does not increase protein abundance that aids in Cu storage and/or proteins that aid in oxidative stress mitigation, which might be one of the factors that contribute to the high fatality from Cu toxicity in sheep. The utilization of radiolabeled Cu molecules in future research could aid in the understanding of Cu homeostasis in sheep. In addition, future research examining Cu homeostasis in the jejunum and ileum of the sheep is warranted.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ruminants6020035/s1>, Figure S1: Principal component analysis of duodenum proteome across dietary copper treatments. Condition: Control = sheep received no supplemental Cu for 29 days. Treatment = sheep received 12 mg of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /kg DM for 29 days; Figure S2: Principal component analysis of liver proteome across dietary copper treatments. Condition: Control = sheep received no supplemental Cu for 29 days. Treatment = sheep received 12 mg of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /kg DM for 29 days; Figure S3: Protein abundance analysis of duodenum and liver proteome across dietary copper treatments. Group: Control = sheep received no supplemental Cu for 29 days. Treatment = sheep received 12 mg of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /kg DM for 29 days; Table S1: Proteins that were exclusively present in liver tissue of control sheep; Table S2: Proteins that were exclusively present in liver tissue of the +Cu sheep; Table S3: Proteins that were exclusively present in duodenum of the control sheep; Table S4: Proteins that were exclusively present in duodenal tissues of +Cu sheep.

Author Contributions: Funding acquisition, T.E.E.; methodology, H.Y.L. and T.E.E.; investigation, H.Y.L. and T.E.E.; formal analysis, H.Y.L.; data curation, H.Y.L.; conceptualization, H.Y.L. and T.E.E.; writing—original draft, H.Y.L.; writing—review and editing, H.Y.L. and T.E.E.; supervision, T.E.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Colorado State University Agricultural Experiment Station (funding number: 1592167).

Institutional Review Board Statement: The animal study protocol was approved by Colorado State University Animal Care and Use Committee (protocol code 4293 and date of approval: 8 March 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The MS proteomics data (Raw and McTab data) have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>, accessed on 9 May 2025) via the PRIDE [64] partner repository with the data set identifier PXD063823.

Acknowledgments: We acknowledge ARDEC employees (Micah Schmidt and Christina Nash) and student researchers (Arturo Rodezno Gomez, Alejandro Quevedo, and Ashlee Gibbons) for their assistance in animal feeding and care. We also acknowledge Colorado State University Analytical Resources Core (SCR_021758) and their team members (Gustavo Diaz, Corey Broeckling, and Dorathea Lee) for their help in the proteomic analysis of samples. We thank Mahesh Nair and Chaoyu Zhai for providing their expertise and advice on methodology and reviewing the final version of the paper.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ATOX1	Antioxidant 1
ATP7A	Menkes protein
ATP7B	Wilson disease protein

BW	Body weight
BH	Benjamini–Hochberg
CCS	Cu chaperone for superoxide dismutase
CCO	Cu cytochrome c oxidase
COX17	Cyclo-oxygenase 17
CTR1	Cu transporter protein 1
Cybrd1	Cytochrome B reductase 1
DMI	Dry matter intake
FDR	False discovery rate
GSH	Glutathione
GSS	Glutathione synthetase
PBS	Phosphate-buffered saline
PSM	Peptide spectral matches
Mt	metallothionein
ROS	reactive oxygen species
SOD	superoxide dismutase
XIAP	X-linked inhibitor of apoptosis protein

References

- Harris, E.D. Copper Transport: An Overview. *Proc. Soc. Exp. Biol. Med.* **1991**, *196*, 130–140. [\[CrossRef\]](#)
- Peña, M.M.O.; Lee, J.; Thiele, D.J. A Delicate Balance: Homeostatic Control of Copper Uptake and Distribution. *J. Nutr.* **1999**, *129*, 1251–1260. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wintrobe, M.M.; Cartwright, G.E.; Gubler, C.J. Studies on the Function and Metabolism of Copper. *J. Nutr.* **1953**, *50*, 395–419. [\[CrossRef\]](#) [\[PubMed\]](#)
- Borobia, M.; Villanueva-Saz, S.; Ruiz de Arcaute, M.; Fernández, A.; Verde, M.T.; González, J.M.; Navarro, T.; Benito, A.A.; Arnal, J.L.; De las Heras, M.; et al. Copper Poisoning, a Deadly Hazard for Sheep. *Animals* **2022**, *12*, 2388. [\[CrossRef\]](#)
- Todd, J.R. Chronic Copper Toxicity of Ruminants. *Proc. Nutr. Soc.* **1969**, *28*, 189–198. [\[CrossRef\]](#)
- Gummow, B.; Botha, C.J.; Basson, A.T.; Bastianello, S.S. Copper Toxicity in Ruminants: Air Pollution as a Possible Cause. *Onderstepoort J. Vet. Res.* **1991**, *58*, 33–39.
- Gooneratne, S.R.; Howell, J.M.; Gawthorne, J. Intracellular Distribution of Copper in the Liver of Normal and Copper Loaded Sheep. *Res. Vet. Sci.* **1979**, *27*, 30–37. [\[CrossRef\]](#)
- López-Alonso, M.; Prieto, F.; Miranda, M.; Castillo, C.; Hernández, J.R.; Benedito, J.L. Intracellular Distribution of Copper and Zinc in the Liver of Copper-Exposed Cattle from Northwest Spain. *Vet. J.* **2005**, *170*, 332–338. [\[CrossRef\]](#)
- Suttle, N.F. *Mineral Nutrition of Livestock*, 4th ed.; CABI North American Office: Cambridge, MA, USA, 2010; ISBN 978-1-84593-473-6.
- Gooneratne, S.R.; Laarveld, B.; Chaplin, R.K.; Christensen, D.A. Profiles of ⁶⁷Cu in Blood, Bile, Urine and Faeces from ⁶⁷Cu-Primed Lambs: Effect of ⁹⁹Mo-Labelled Tetrathiomolybdate on the Metabolism of Recently Stored Tissue ⁶⁷Cu. *Br. J. Nutr.* **1989**, *61*, 355–371. [\[CrossRef\]](#)
- Clarkson, A.H.; Paine, S.; Martín-Tereso, J.; Kendall, N.R. Copper Physiology in Ruminants: Trafficking of Systemic Copper, Adaptations to Variation in Nutritional Supply and Thiomolybdate Challenge. *Nutr. Res. Rev.* **2020**, *33*, 43–49. [\[CrossRef\]](#) [\[PubMed\]](#)
- Lee, J.; Peña, M.M.O.; Nose, Y.; Thiele, D.J. Biochemical Characterization of the Human Copper Transporter Ctr1. *J. Biol. Chem.* **2002**, *277*, 4380–4387. [\[CrossRef\]](#)
- Lee, J.; Prohaska, J.R.; Thiele, D.J. Essential Role for Mammalian Copper Transporter Ctr1 in Copper Homeostasis and Embryonic Development. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 6842–6847. [\[CrossRef\]](#) [\[PubMed\]](#)
- Labbé, S.; Thiele, D.J. Pipes and Wiring: The Regulation of Copper Uptake and Distribution in Yeast. *Trends Microbiol.* **1999**, *7*, 500–505. [\[CrossRef\]](#)
- Simpson, D.M.; Beynon, R.J.; Robertson, D.H.L.; Loughran, M.J.; Haywood, S. Copper-Associated Liver Disease: A Proteomics Study of Copper Challenge in a Sheep Model. *Proteomics* **2004**, *4*, 524–536. [\[CrossRef\]](#)
- Simpson, D.M.; Mobasher, A.; Haywood, S.; Beynon, R.J. A Proteomics Study of the Response of North Ronaldsay Sheep to Copper Challenge. *BMC Vet. Res.* **2006**, *2*, 36. [\[CrossRef\]](#)
- Haywood, S.; Müller, T.; Müller, W.; Heinz-Erian, P.; Tanner, M.S.; Ross, G. Copper-Associated Liver Disease in North Ronaldsay Sheep: A Possible Animal Model for Non-Wilsonian Hepatic Copper Toxicosis of Infancy and Childhood. *J. Pathol.* **2001**, *195*, 264–269. [\[CrossRef\]](#) [\[PubMed\]](#)

18. Ishmael, J.; Gopinath, C.; Howell, J.M. Experimental Chronic Copper Toxicity in Sheep: Biochemical and Haematological Studies during the Development of Lesions in the Liver. *Res. Vet. Sci.* **1972**, *13*, 22–29. [[CrossRef](#)]
19. NASEM (National Academies of Sciences, Engineering, and Medicine). *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*; National Academies Press: Washington, DC, USA, 2007; ISBN 978-0-309-10213-1.
20. Han, H.; Archibeque, S.L.; Engle, T.E. Characterization and Identification of Hepatic mRNA Related to Copper Metabolism and Homeostasis in Cattle. *Biol. Trace Elem. Res.* **2009**, *129*, 130–136. [[CrossRef](#)]
21. Loh, H.Y.; Spears, J.W.; Guimaraes, O.; Miller, A.C.; Thorndyke, M.P.; Thomas, T.A.; Engle, T.E. Trace Mineral Source Influences Trace Mineral Solubility in Water and Mineral Binding Strength to Ruminant Digesta. *Biol. Trace Elem. Res.* **2024**, *203*, 1830–1838. [[CrossRef](#)] [[PubMed](#)]
22. Scopes, R.K. Measurement of Protein by Spectrophotometry at 205 nm. *Anal. Biochem.* **1974**, *59*, 277–282. [[CrossRef](#)]
23. Eng, J.K.; McCormack, A.L.; Yates, J.R. An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. *J. Am. Soc. Mass. Spectrom.* **1994**, *5*, 976–989. [[CrossRef](#)]
24. The UniProt Consortium UniProt: The Universal Protein Knowledgebase in 2025. *Nucleic Acids Res.* **2025**, *53*, D609–D617. [[CrossRef](#)] [[PubMed](#)]
25. Käll, L.; Canterbury, J.D.; Weston, J.; Noble, W.S.; MacCoss, M.J. Semi-Supervised Learning for Peptide Identification from Shotgun Proteomics Datasets. *Nat. Methods* **2007**, *4*, 923–925. [[CrossRef](#)]
26. R Core Team. *R: A Language and Environment for Statistical Computing*; Foundation for Statistical Computing: Vienna, Austria, 2023.
27. Ahmad, S.; Jose da Costa Gonzales, L.; Bowler-Barnett, E.H.; Rice, D.L.; Kim, M.; Wijerathne, S.; Luciani, A.; Kandasaamy, S.; Luo, J.; Watkins, X.; et al. The UniProt Website API: Facilitating Programmatic Access to Protein Knowledge. *Nucleic Acids Res.* **2025**, *53*, W547–W553. [[CrossRef](#)] [[PubMed](#)]
28. Agri-Food & Biosciences Institute. AFBI Warns of the Risk of Chronic Copper Poisoning in Sheep. Available online: <https://www.afbini.gov.uk/news/afbi-warns-about-risk-chronic-copper-poisoning-sheep> (accessed on 5 March 2025).
29. Suttle, N.F. Copper Imbalances in Ruminants and Humans: Unexpected Common Ground. *Adv. Nutr.* **2012**, *3*, 666–674. [[CrossRef](#)]
30. Nevitt, T.; Öhrvik, H.; Thiele, D.J. Charting the Travels of Copper in Eukaryotes from Yeast to Mammals. *Biochim. Biophys. Acta (BBA)—Mol. Cell Res.* **2012**, *1823*, 1580–1593. [[CrossRef](#)]
31. López-Alonso, M.; Miranda, M. Copper Supplementation, A Challenge in Cattle. *Animals* **2020**, *10*, 1890. [[CrossRef](#)]
32. Knöpfel, M.; Solioz, M. Characterization of a Cytochrome B558 Ferric/Cupric Reductase from Rabbit Duodenal Brush Border Membranes. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 220–225. [[CrossRef](#)]
33. Tennant, J.; Stansfield, M.; Yamaji, S.; Srai, S.K.; Sharp, P. Effects of Copper on the Expression of Metal Transporters in Human Intestinal Caco-2 Cells. *FEBS Lett.* **2002**, *527*, 239–244. [[CrossRef](#)] [[PubMed](#)]
34. Prohaska, J.R. Role of Copper Transporters in Copper Homeostasis. *Am. J. Clin. Nutr.* **2008**, *88*, 826S–829S. [[CrossRef](#)]
35. Linder, M.C.; Zerounian, N.R.; Moriya, M.; Malpe, R. Iron and Copper Homeostasis and Intestinal Absorption Using the Caco2 Cell Model. *Biomaterials* **2003**, *16*, 145–160. [[CrossRef](#)]
36. Maryon, E.B.; Molloy, S.A.; Kaplan, J.H. Cellular Glutathione Plays a Key Role in Copper Uptake Mediated by Human Copper Transporter 1. *Am. J. Physiol.-Cell Physiol.* **2013**, *304*, C768–C779. [[CrossRef](#)] [[PubMed](#)]
37. Dameron, C.T.; Harrison, M.D. Mechanisms for Protection against Copper Toxicity. *Am. J. Clin. Nutr.* **1998**, *67*, 1091S–1097S. [[CrossRef](#)]
38. Weiss, K.C.; Linder, M.C. Copper Transport in Rats Involving a New Plasma Protein. *Am. J. Physiol.-Endocrinol. Metab.* **1985**, *249*, E77–E88. [[CrossRef](#)] [[PubMed](#)]
39. Bremner, I.; Beattie, J.H. Copper and Zinc Metabolism in Health and Disease: Speciation and Interactions. *Proc. Nutr. Soc.* **1995**, *54*, 489–499. [[CrossRef](#)]
40. La Fontaine, S.; Mercer, J.F.B. Trafficking of the Copper-ATPases, ATP7A and ATP7B: Role in Copper Homeostasis. *Arch. Biochem. Biophys.* **2007**, *463*, 149–167. [[CrossRef](#)] [[PubMed](#)]
41. Ferenci, P.; Caca, K.; Loudianos, G.; Mieli-Vergani, G.; Tanner, S.; Sternlieb, I.; Schilsky, M.; Cox, D.; Berr, F. Diagnosis and Phenotypic Classification of Wilson Disease. *Liver Int.* **2003**, *23*, 139–142. [[CrossRef](#)]
42. de Bie, P.; van de Sluis, B.; Klomp, L.; Wijmenga, C. The Many Faces of the Copper Metabolism Protein MURR1/COMMD1. *J. Hered.* **2005**, *96*, 803–811. [[CrossRef](#)]
43. Collins, J.F.; Prohaska, J.R.; Knutson, M.D. Metabolic Crossroads of Iron and Copper. *Nutr. Rev.* **2010**, *68*, 133–147. [[CrossRef](#)]
44. Lin, C.; Zhang, Z.; Wang, T.; Chen, C.; James Kang, Y. Copper Uptake by DMT1: A Compensatory Mechanism for CTR1 Deficiency in Human Umbilical Vein Endothelial Cells. *Metallomics* **2015**, *7*, 1285–1289. [[CrossRef](#)]
45. Zimnicka, A.M.; Ivy, K.; Kaplan, J.H. Acquisition of Dietary Copper: A Role for Anion Transporters in Intestinal Apical Copper Uptake. *Am. J. Physiol. Cell Physiol.* **2011**, *300*, C588–C599. [[CrossRef](#)]
46. Lönnerdal, B. Intestinal Regulation of Copper Homeostasis: A Developmental Perspective1. *Am. J. Clin. Nutr.* **2008**, *88*, 846S–850S. [[CrossRef](#)]

47. Horng, Y.-C.; Cobine, P.A.; Maxfield, A.B.; Carr, H.S.; Winge, D.R. Specific Copper Transfer from the Cox17 Metallochaperone to Both Sco1 and Cox11 in the Assembly of Yeast Cytochrome c Oxidase. *J. Biol. Chem.* **2004**, *279*, 35334–35340. [CrossRef]
48. Li, H.; Du, X.; Li, X.; Feng, P.; Chu, M.; Jin, Y.; Pan, Z. Genetic Diversity, Tissue-Specific Expression, and Functional Analysis of the ATP7A Gene in Sheep. *Front. Genet.* **2023**, *14*, 1239979. [CrossRef] [PubMed]
49. Anderson, M. Mn Ions Pass through Calcium Channels. A Possible Explanation. *J. Gen. Physiol.* **1983**, *81*, 805–827. [CrossRef]
50. Söli, N.E.; Rambæk, J.P. Excretion of Intravenously Injected Copper-64 in Sheep. *Acta Pharmacol. Toxicol.* **1978**, *43*, 205–210. [CrossRef] [PubMed]
51. Deol, H.S.; Howell, J.M.; Dorling, P.R.; Symonds, H.W. The Effect of Copper and Heliotrope on the Composition of Bile in Sheep. *Res. Vet. Sci.* **1992**, *53*, 324–330. [CrossRef]
52. Minervino, A.H.H.; López-Alonso, M.; Barrêto Júnior, R.A.; Rodrigues, F.A.M.L.; Araújo, C.A.S.C.; Sousa, R.S.; Mori, C.S.; Miranda, M.; Oliveira, F.L.C.; Antonelli, A.C.; et al. Dietary Zinc Supplementation to Prevent Chronic Copper Poisoning in Sheep. *Animals* **2018**, *8*, 227. [CrossRef]
53. Bremner, I.; Mehra, R.K.; Morrison, J.N.; Wood, A.M. Effects of Dietary Copper Supplementation of Rats on the Occurrence of Metallothionein-I in Liver and Its Secretion into Blood, Bile and Urine. *Biochem. J.* **1986**, *235*, 735–739. [CrossRef] [PubMed]
54. Aishajiang, R.; Liu, Z.; Wang, T.; Zhou, L.; Yu, D. Recent Advances in Cancer Therapeutic Copper-Based Nanomaterials for Antitumor Therapy. *Molecules* **2023**, *28*, 2303. [CrossRef] [PubMed]
55. Saylor, W.W.; Leach, R.M. Intracellular Distribution of Copper and Zinc in Sheep: Effect of Age and Dietary Levels of the Metals. *J. Nutr.* **1980**, *110*, 448–459. [CrossRef] [PubMed]
56. Qin, C.; Qiu, K.; Sun, W.; Jiao, N.; Zhang, X.; Che, L.; Zhao, H.; Shen, H.; Yin, J. A Proteomic Adaptation of Small Intestinal Mucosa in Response to Dietary Protein Limitation. *Sci. Rep.* **2016**, *6*, 36888. [CrossRef]
57. Puls, R. *Mineral Levels in Animal's Health: Diagnostic Data*; Sherpa International: Clearbrook, BC, Canada, 1994; Volume 83.
58. Blakley, B.R. Copper Poisoning in Animals—Toxicology. Available online: <https://www.merckvetmanual.com/toxicology/copper-poisoning/copper-poisoning-in-animals> (accessed on 12 March 2025).
59. Dezfoulan, A.H.; Aliarabi, H.; Tabatabaei, M.M.; Zamani, P.; Alipour, D.; Bahari, A.; Fadayifar, A. Influence of Different Levels and Sources of Copper Supplementation on Performance, Some Blood Parameters, Nutrient Digestibility and Mineral Balance in Lambs. *Livest. Sci.* **2012**, *147*, 9–19. [CrossRef]
60. UniProt. Available online: <https://www.uniprot.org> (accessed on 12 March 2025).
61. McEwan, D.G.; Ryan, K.M. ATG2 and VPS13 Proteins: Molecular Highways Transporting Lipids to Drive Membrane Expansion and Organelle Communication. *FEBS J.* **2022**, *289*, 7113–7127. [CrossRef]
62. Li, S.; Liu, Y.; Bai, Y.; Chen, M.; Cheng, D.; Wu, M.; Xia, J. Ras Homolog Family Member F, Filopodia Associated Promotes Hepatocellular Carcinoma Metastasis by Altering the Metabolic Status of Cancer Cells Through RAB3D. *Hepatology* **2021**, *73*, 2361–2379. [CrossRef]
63. Zhu, X.; Zhu, P.; Chen, X.; Zhang, L.; Wu, C.; Zhang, H.; Shen, X.; Qi, Y.; Chen, M.; Wang, S.; et al. Correlation of 3'-Phosphoadenosine-5'-Phosphosulfate Synthase 1 (PAPSS1) Expression with Clinical Parameters and Prognosis in Esophageal Squamous Cell Carcinoma. *Histol. Histopathol.* **2023**, *38*, 1327–1335. [CrossRef]
64. Perez-Riverol, Y.; Bandla, C.; Kundu, D.J.; Kamatchinathan, S.; Bai, J.; Hewapathirana, S.; John, N.S.; Prakash, A.; Walzer, M.; Wang, S.; et al. The PRIDE Database at 20 Years: 2025 Update. *Nucleic Acids Res.* **2025**, *53*, D543–D553. [CrossRef] [PubMed]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.