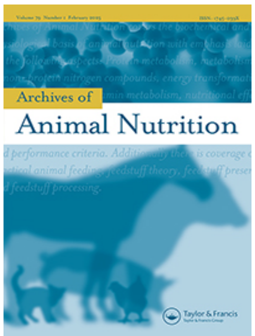

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Influence of copper level and source on performance, tissue accumulation, and faecal excretion in fattening pigs

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ABSTRACT

Copper (Cu) supplementation is essential in pig nutrition; however, its effects on performance, trace element accumulation in edible tissues, and environmental excretion require careful evaluation. In the present study a total of 24 male, castrated fattening pigs of two different hybrid mast lines (11 weeks of age) were divided according to their initial body weight (25.8 ± 3.5 kg) into four groups. Pigs were fed for 14 weeks a complete feed supplemented with Cu covering a range between the recommended Cu supplementation and the permissible European maximum level (i.e. 5, 15, and 25 mg/kg complete feed). Two different Cu sources were used: Cu sulphate ($CuSO_4$) and glycine-Cu chelate hydrate (Cu-Gly). The aim of the trial was studying the influence of the different Cu levels and sources on growth performance, Cu transfer to edible tissues (muscle, liver, kidney, blood) and faeces, as well as the concentration of other trace elements, including iron (Fe), zinc (Zn) and manganese (Mn) in tissues of fattening pigs. Performance parameters, including average daily gain (ADG) and feed conversion ratio (FCR), showed a dependency with respect to the pig breed, whereas Cu content and Cu sources showed no influence. Copper concentrations in the liver, kidney, muscle, and blood serum remained constant across groups. Faecal Cu excretion increased proportionally with dietary Cu levels, with higher excretion observed for the organic Cu source at 15 mg/kg as compared with inorganic source at similar level. The results show, that Cu levels up to the maximum level of 25 mg/kg complete feed are not necessary to achieve good growth performance in healthy fattening pigs. The unaffected Cu concentrations in liver, muscle, kidney, and blood, as well as increased faecal excretion with increasing Cu level in the feed, indicate an adequate supply and homoeostatic regulation of Cu. In addition, a reduced use of Cu in pig fattening will help to reduce Cu emission into the environment.

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1. Introduction

As an essential trace element, copper (Cu) plays numerous roles in the organism. Its importance as a component of enzymes, in oxygen transport, in protection against oxidative stress, as well as its involvement in cellular respiration and tissue pigmentation is well known (Espinosa and Stein 2021). In the body, Cu is stored in the liver and kidneys and excreted primarily via the bile and finally faeces. The German Society for Nutrition Physiology (GfE) recommends a Cu supply of 4–5 mg/kg of complete feed for fattening pigs (GfE 2006). Dietary components such as zinc, iron and phytates may have an antagonistic effect with possible increase in Cu requirement (EFSA 2016). Since most feed materials used in animal nutrition are low in Cu, supplementation is usually accomplished via feed additives. However, a maximum permissible Cu levels in complete feed in the European Union (EU) exits. For fattening pigs, the maximum content is currently 25 mg Cu per kg complete feed of 12% moisture (EU 2018). For piglets up to eight weeks after weaning, a Cu supply that exceeds requirements is permitted in feed (100–150 mg/kg depending on age; EU 2018), to use the pharmacological effects of the element. High Cu doses in early weeks of life positively impact the intestinal health of piglets, which in turn can increase feed intake and body weight gain (Espinosa and Stein 2021; Forouzandeh et al. 2022). Compared to ruminants, especially sheep, which are the most sensitive species to Cu intoxication, pigs are very tolerant to high Cu intakes (Lopez-Alonso 2012). While 12–36 mg Cu/kg dry matter (DM) already leads to symptoms of poisoning in sheep (Suttle 2022), pigs show a significant increase in Cu storage in the liver at levels of up to 250 mg/kg complete feed without negative effects on health or performance (EFSA 2016). However, high Cu supplementation in feed promote the transfer into food of animal origin. In the National Residue Control Plan (NRCP), samples, primarily from liver, are repeatedly found to be non-compliant with regard to Cu content (e. g. NRCP 2021) and exceeding the maximum residue level of 30 mg/kg for liver of farmed animals (EC 2005). Investigations of the German Total Diet Study indicated, that frequent adult human consumers of mammalian liver exceed the acceptable daily intake (ADI) of 0.15 mg Cu per kg body weight per day, based on median and 95th percentile intake level (Kolbaum et al. 2023). In addition to elevated Cu levels in food of animal origin, high Cu intake in pig farming also poses environmental concerns, as excess Cu is excreted in the faeces and subsequently enters the soil as liquid manure (Mallmann et al. 2014; Brugger and Windisch 2015). Bacterial heavy metal tolerance (Cu resistance) and resistance to certain antibiotics are being discussed (Yazdankhah et al. 2014). In 2005, Cu input into the soil in Germany was about 3,600 t, with agricultural manure identified as the main source (Hillenbrand et al. 2005). However, pesticides used in both conventional and organic farming also contribute to the accumulation of Cu in the soil. Over the past 20 years, measures have been implemented within the European Union to limit the use of Cu in pesticide management and animal feed with the aim to reduce the Cu input into the soil. In animal nutrition, Cu is primarily used as an inorganic compound, commonly in the form of Cu sulphate ($CuSO_4$). Copper from organic sources (e. g. bound as chelate or to amino acids) is often described to have better bioavailability, with positive impacts on performance parameters, especially in piglet nutrition with high Cu concentrations of up to 200 mg/kg of complete feed (Coffey et al. 1994; Zhao et al. 2014).

In the present study, fattening pigs were fed different Cu levels in their diet ranging between an aimed concentration of 5 to 25 mg/kg of complete feed, with two different Cu sources being tested for a dosage of 15 mg/kg. Typical performance parameters in relation to Cu feed level and source were assessed, as well as Cu transfer to animal matrices (tissue of muscle, liver and kidney as well as blood serum) and Cu excretion levels in faeces. The aim of the study was to demonstrate, that reaching the maximum permissible Cu content in feed is not necessary to achieve a good performance in healthy pigs. Our results may contribute to establishing legally binding regulations to reduce the use of Cu in pig nutrition. Reduced Cu supplementation in pigs can have a positive impact on the environment.

2. Materials and methods

2.1. Animals and dietary treatment

A total of 24 male castrated pigs (11 weeks of age, 25.8 ± 3.5 kg BW, German Saddleback ×Pietrain ($n = 15$) or German Landrace ×Pietrain ($n = 9$)), origin: Oberholz Farm for Teaching and Research, Faculty of Veterinary Medicine, Leipzig University) were randomly allocated into four groups of six animals each according to their weight on the research station of the German Federal Institute for Risk Assessment (BfR). Both pig breeds were represented in the four feeding groups, but unevenly. Each pen (30 m^2) was equipped with a rubber lying mat and sawdust as bedding material in the lying area, providing enough space for all pigs lying simultaneously. Enrichment materials (straw rack, balls, tubes) were provided according to current recommendations (EU 2009, 2016). The pigs were fed according to a restrictive feeding plan, based on their metabolic body weight (110 g/kg $BW^{0.75}$ per day). Feed in mash form was provided as meal twice daily. The servings per animal were distributed at a distance in the trough (trough length for 6 animals: 8 m) in order to achieve the most separate feed intake possible. This allowed undisturbed feeding for all animals at times. Backweighing was not necessary as the troughs were empty. However, the ingestion of residual feed by other animals of the group cannot be prevented. Water was available *ad libitum* via nipple drinkers.

To acclimatise the pigs to the new situation (e. g. new feed, changed feeding times) after their arrival, all pigs received the same ration during a one-week adaptation period, with no additional Cu added to the basal ration. After the adaptation period, each of the four groups was given a different ration. Three different Cu concentrations in the complete feed should be achieved: Cu-low = 5 mg/kg, representing the GfE supply recommendation; Cu-medium = 15 mg/kg (two groups), typical for common farming practice; and Cu-high = 25 mg/kg, representing the maximum concentration according to European Regulation (EU 2018). The complete feed for the pigs in the Cu-low and Cu-high groups was enriched with an inorganic Cu feed additive (Cu(II) sulphate pentahydrate; $CuSO_4 \cdot 5\,H_2O$, 25% Cu, Norkem, Knutsford, England). The Cu-medium groups of 15 mg/kg received either the inorganic preparation or an organic Cu preparation (B-Traxim, 2C Cu – 240: glycine Cu chelate hydrate, 24% Cu, Pancosma, Rolle, Switzerland; short: Cu-Gly). The planed and actual Cu contents in the individual rations are shown in Table 1.

Table 1. Ingredients and analysed composition of the experimental diets with copper (Cu).

	Basal diet	Cu-low	Cu-medium	Cu-medium	Cu-high
aimed Cu content [mg/kg complete feed]	-	5	15	15	25
Cu source[1]	-	$CuSO_4$	$CuSO_4$	Cu-Gly	$CuSO_4$
Ingredients [g/kg complete feed, 88% DM]					
Barley	330	330	330	330	330
Wheat	380	378	379	370	370
Corn	0	0.995	0.970	0.970	0.930
Soy extraction meal	250	250	250	250	250
Plant oil	20	20	20	20	20
Vitamin-trace mineral mix (without Cu)[2]	10	10	10	10	10
Limestone	10	10	10	10	10
CuSO4	0	0.005	0.030	0	0.070
Cu-Gly	0	0	0	0.030	0
Analysed composition [g/kg, 88% DM]					
DM [g/kg fresh matter]	869	897	899	899	898
Crude ash	41	49	45	43	46
Crude protein	155	185	172	173	175
Crude fibre	45	45	45	44	43
Starch	431	381	407	401	402
Sugar	40	39	36	38	38
Crude fat	37	41	40	38	36
Acid detergent fibre (ADF)	50	62	58	55	62
Neutral detergent fibre (NDF)	140	153	148	151	158
ME (MJ)	13.3	13.1	13.2	13.2	13.0
Analysed (trace) elements					
g/kg, 88% DM					
Ca	6.0	7.0	5.6	5.8	6.2
P	3.2	3.8	3.6	3.5	3.5
Na	1.2	1.1	0.9	1.0	0.9
Mg	1.9	2.7	2.3	2.3	2.2
K	7.2	9.2	8.1	7.7	8.0
mg/kg, 88% DM					
Cu	5.2	9.3	14.2	12.9	25.4
Mn	67.8	76.0	58.5	76.2	59.6
Zn	93.8	130.6	78.1	102.5	82.7
Fe	222.0	255.6	232.1	231.6	233.0

[1]Cu Source (abbreviation): $CuSO_4$ = Cu(II) sulphate pentahydrate, $CuSO_4 \cdot 5\,H_2O$, 25% Cu; Cu-Gly = glycine Cu chelate hydrate, 2C Cu − 240, 24% Cu.
[2]Composition of the premix (without Cu supplementation; per kg of diet): vitamin A, 5000 IU; vitamin D3, 1200 IU; vitamin E, 80 mg; vitamin K3, 3 mg; vitamin B1, 2.5 mg; vitamin B2, 2.5 mg; vitamin B6, 4 mg; vitamin B12, 20 mg; Niacin, 25 mg; biotin, 250 mg; folic acid, 1 mg; pantothenic acid, 10 mg; choline chloride, 500 mg; iron (sulfat), 50 mg; zinc (oxid), 50 mg; mangan (oxid), 60 mg; iodine (calcium), 0.45 mg; and selenium (selenite), 0.35 mg.

The composition of the rations, as well as the concentrations of crude nutrients and (trace) elements in the complete feed are shown in Table 1. Representative samples of each feedingstuff were taken at two different time points during the trial and sent to a DIN EN ISO/IEC 17,025:2018 accredited laboratory (Landeskontrollverband Berlin-Brandenburg e.V.) for analysis. The crude ash (XA, 8.1), crude fat (XL, 5.1.1), crude protein (XP, 4.1.1), crude fibre (XF, 6.1.1), starch (7.2.1), sugar (7.1.1), acid detergent fibre (ADF, 6.5.2) and neutral detergent fibre (NDF, 6.5.1) contents as well as the contents of (trace) elements (10.8.2) were determined according to the VDLUFA (2012). The results of the feed analysis are represented as the mean value of the two individual measurement. For some trace elements, like zinc, deviations in the contents between the groups were subsequently found, which are presumably due to segregation. The animals were fattened for a total of 100 days at the BfR before being slaughtered by electrical stunning and exsanguination. Slaughter, conducted externally, was supervised by an official veterinarian from the federal state of Brandenburg.

2.2. Growth performance and sampling

All pigs were weighed at the beginning and weekly thereafter to calculate the average daily gain (ADG) and feed conversion rate (FCR). A pooled faecal sample was collected weekly from each group and frozen at −20°C until analysis. Contamination of the faecal samples was minimised by the absence of bedding material in the manure area and appropriate sampling. During slaughter, tissue samples were collected from the liver (peripheral tissue from the left lateral lobe), muscle tissue (foreleg, knuckle), kidney and blood. Blood samples were centrifuged (3000 U/min, 20°C, 10 min) and resulting serum along with muscle, liver and kidney tissue was deep-frozen at − 20°C until further use.

2.3. Elemental analysis of liver, muscle, kidney and blood serum samples

Copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) concentrations were quantified in the liver, muscle, kidney and blood serum samples by inductively-coupled plasma tandem mass spectrometry (ICP-MS/MS). The analyses were conducted by the Department of Food Chemistry at the University of Potsdam.

Serum trace elements (TEs) were quantified based on a well-established multi-element protocol (Kopp et al. 2019). A 50 µL serum aliquot was diluted 1:10 with 430 µL of matrix mix containing 1% -butanol (Alfa Aesar), 0.05% Na-EDTA (Triplex® III, pro Analysis, Merck), 0.05% Triton™ X-100 (10% in H_2O, Merck-Sigma Aldrich), and 0.25% ammonium hydroxide (NH_4OH, 25% in H_2O, Fluka). Additionally, 20 µL of an internal standard mix (250 µg/L germanium (Ge), 25 µg/L rhodium (Rh) (both, Carl Roth) and 250 µg/L ^{77}Se (^{77}Se 97.20 ± 0.20%; ^{74}Se 0.10%; ^{76}Se 0.40 ± 0.10%; ^{78}Se 2.40 ± 0.10%; ^{80}Se 0.10%; ^{82}Se 0.10%, certified by Trace Sciences International, Ontario, Canada) were added to the samples, resulting in a final concentration of 1 µg/L Rh, 10 µg/L Ge, 10 µg/L ^{77}Se. For Mn, Fe, Cu, and Zn external calibrations were prepared from 1 g/L standard solutions (Carl Roth, Inorganic Ventures, or VHG Labs). During each analysis two replicates of concentration level I and II of ClinChek® reference serum (RECIPE Chemicals + Instruments and Seronorm™; Lot 2062) were prepared like the samples for quality control and analysed by ICP-MS/MS (Agilent ICP-QQQ-MS 8800, Agilent Technologies, Waldbronn, Germany).

For tissue TE quantification about 30 to 50 mg of frozen tissue from the six animals per group, were weighed into polytetrafluoroethylene (PTFE) microwave vessels. Microwave-assisted acid digestion was performed as described before (Lossow et al. 2020). Briefly, 900 µL of nitric acid (HNO_3; 65% Triplex®, Merck), 250 µL of H_2O_2 (30%, Merck), 200 µL of an internal standard mix, containing 100 µg/L Rh, 1000 µg/L Ge and 1000 µg/L ^{77}Se (same stocks as for the serum analysis) and 650 µL of ultrapure water were added to each vessel. Quality control included the digestion of three reagent blanks and two replicates (30 mg each) of ERM-BB422 (fish muscle) and ERM-BB186 (pig kidney) certified reference materials (IRMM, Geel, Belgium). After microwave digestion, the samples were diluted 1:5 to a final concentration of 1 µg/L Rh, 10 µg/L Ge, 10 µg/L ^{77}Se, 4.5% (v/v) HNO_3 and analysed by ICP-MS/MS. For Mn (VHG Labs, Manchester, New Hampshire, USA), Fe, Cu and Zn (Carl Roth, Karlsruhe, Germany) an external calibration was prepared from 1 g/L stock solutions. The limits of detection (LOD) and quantification (LOQ) were calculated as the mean concentration of at least three technical blanks plus three times the corresponding standard deviation (SD) for the LOD, or plus ten times SD for the LOQ.

2.4. Copper analysis in faeces

Prior to analysis, pig faeces samples were homogenised and dried at 70°C for 72 hours in an ED Series drying oven (Binder, Tuttlingen, Germany). Approximately 100 mg of the dried faecal sample was weighed using an XP series analytical balance (Mettler Toledo, Columbus, OH, USA) and placed into pre-cleaned 18 mL PTFE tubes (Anton Paar, Graz, Austria). Subsequently, 3 mL of concentrated HNO_3, double-distilled using a subCLEAN system (Milestone, Sorisole, Italy), was added. Samples were digested using the MultiWave 7000 system (Anton Paar) at 220°C, with a 10-minute ramp, 20-minute hold, and 10-minute cool-down period. After digestion, the samples were quantitatively transferred to 14 mL conical tubes, diluted to a total volume of 10 mL, and further diluted with 6% HNO_3 as needed to fit within the calibration range.

Copper analysis was conducted using graphite furnace atomic absorption spectrometry (GF-AAS) with the PinAAcle 900Z (PerkinElmer, Waltham, MA, USA) and a 5-point external calibration curve (0.005–0.070 mg/L) prepared from a 1000 mg/L Cu stock solution (Merck, Darmstadt, Germany). For each measurement, 20 µl of the digested sample were supplemented by 5 µL matrix modifier (0.015 mg $Mg(NO_3)_2$) and 5 µL blank solution (6.9% HNO_3/water). The temperature cycle programme contained the following steps: Dry1 (110°C, 30 s), Dry2 (130°C, 45 s), Pyrolysis (1200°C, 30 s), Atomisation (2000°C, 5 s), Cleanout (2450°C, 4 s). A zeeman background correction was applied. Quality control of the measurement included reagent blanks, certified reference material ERM-BB185 bovine liver (IRMM, Geel, Belgium), and samples spiked with 0.010 mg of Cu. The mean Cu recovery for ERM-BB185 ($N = 4$) was 104%, and the mean spiking recovery ($N = 11$) was 95%. The limit of quantification (LOQ) was 0.005 mg/L. Copper concentrations in faeces are reported as mg/kg dry weight.

2.5. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Data for performance and Cu concentrations in the faeces were subjected to analysis of variance (ANOVA, GLM procedure). Data for the concentrations of Cu, Fe, Zn and Mn in liver, muscle, kidney and blood serum were analysed as a randomised design using the MIXED model procedure (with the breed as random factor). Fixed factors included group (comparing all four feeding groups), dosage (comparing the three feeding groups of different Cu dosage, supplemented with $CuSO_4$) and source (comparing of the two Cu-medium groups supplemented either with $CuSO_4$ or Cu-Gly). Significant differences were declared at $p < 0.05$.

Manganese concentrations in blood serum and muscle were very low and in the case of muscle tissue always below the LOQ. Therefore, Mn levels in serum and muscle were excluded form statistical analysis. Additionally, one liver sample from the Cu-high group was unusable, resulting in a total of 332 measured values available for evaluation of Cu, Fe, Mn and Zn in liver, muscle, kidney and blood serum. A total of 56 faecal samples were analysed for Cu content. Within the whole data set, five outliers were identified (Boxplot) and removed, to ensure normal distribution of the data.

In addition, correlations between the Cu content in the feed and the measured values or between the individual trace element concentrations in the different tissues was examined (Pearson correlation coefficient; Microsoft Excel, Version 16.0, Microsoft Corporation, 2021).

3. Results

All pigs remained healthy throughout the study and no veterinary treatment was required.

Table 1 presents a comparison of the planed and actual Cu levels in the feed rations. The native Cu content in the basal diet, which was given to all animals for acclimatisation in the first week of the trial, was 5.2 mg/kg complete feed. In the Cu-low group, the targeted Cu content of 5 mg/kg was exceeded and amounted to 9.3 mg/kg. For the animals in the Cu-medium (15 mg/kg) and Cu-high (25 mg/kg) groups, the desired Cu levels in the respective rations were achieved satisfactorily (Cu-medium: 14.2 and 12.9 mg/kg respectively; Cu-high: 25.4 mg/kg).

3.1. Growth performance

Performance parameters are summarised in Table 2, showing significant influence regarding the breed. Landrace-hybrids had a significantly higher final weight than Saddleback-hybrids (121 kg and 110 kg, respectively; $p = 0.030$). Even ADG and FCR differed significantly with positive impacts for the Landrace-hybrids (ADG: 979 g and 863 g; FCR: 2.6 and 2.9, respectively). Neither the Cu dosage nor the source had an effect on body weight, ADG and FCR. The time was also significant, with body weight, ADG and FCR increased significantly over the 14-week fattening period ($p < 0.001$).

Table 2. Growth performance (mean ± SD) of pigs fed experimental diets with three different levels and two different sources of copper (Cu) during 14 weeks of fattening.

		BW [kg]				
Group[1], Cu dosage	Cu source[2]	Start	End	Gain	ADG [g]	FCR[3]
Cu-low, 5 mg/kg	$CuSO_4$	25.5 ± 3.0	115.3 ± 16.4	89.8 ± 14.0	916 ± 213	2.60 ± 0.40
Cu-medium, 15 mg/kg	$CuSO_4$	26.0 ± 4.5	115.9 ± 13.3	89.9 ± 9.3	917 ± 215	2.63 ± 0.53
Cu-medium, 15 mg/kg	Cu-Gly	25.9 ± 3.5	113.2 ± 6.5	87.3 ± 4.0	891 ± 221	2.72 ± 0.58
Cu-high, 25 mg/kg	CuSO4	25.8 ± 3.0	113.9 ± 8.8	88.1 ± 7.2	899 ± 243	2.70 ± 0.48
	SEM	0.74	2.49	1.97	20.1	0.07
p-value	Time (14 weeks)			**< 0.0001**	**< 0.0001**	**0.0002**
	Group	0.997	0.982	0.962	0.986	0.904
	Breed	0.976	**0.030**	**0.005**	**0.005**	**0.012**
	Source		0.689	0.580	0.765	0.587
	Dosage		0.971	0.956	0.977	0.859

[1]Group = The aimed amount of Cu in a complete feed. [2]Cu source = $CuSO_4$: Cu(II) sulphate pentahydrate; Cu-Gly: glycine Cu chelate hydrate; [3]FCR: calculation is based on the assumption that the feed intake was the same for all animals, divided by the individual animal live weight gain. Abbreviation: BW = body weight; ADG = average daily gain; FCR = feed conservation ratio; SEM = standard error of the mean.

Table 3. Mean concentrations (±SD) of copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn) in different tissues of pigs fed experimental diets with three different levels and two different sources of Cu after 14 weeks of fattening.

			Group[1]/Cu source[2]							
								p-Value		
Tissue	Element[3]	Unit[4]	Cu-low 5 mg/kg $CuSO_4$	Cu-medium 15 mg/kg $CuSO_4$	Cu-medium 15 mg/kg Cu-Gly	Cu-high 25 mg/kg $CuSO_4$	SEM	Group	Source	Dosage
Muscle [mg/kg]	Cu	mg/kg	0.68 ± 0.2	0.84 ± 0.3	0.70 ± 0.3	0.79 ± 0.2	0.05	0.663	0.410	0.519
	Fe		13.7 ± 4.0	15.4 ± 3.7	13.6 ± 3.4	14.9 ± 3.1	0.68	0.814	0.415	0.767
	Zn		40.8 ± 12.6	45.5 ± 9.2	38.2 ± 10.7	43.6 ± 6.7	1.95	0.653	0.266	0.733
Liver [mg/kg]	Cu	mg/kg	7.7 ± 0.6	9.1 ± 2.1	9.3 ± 1.4	7.8 ± 1.0	0.30	0.224	0.855	0.222
	Fe		199 ± 28	176 ± 48	189 ± 26	176 ± 31	6.98	0.613	0.584	0.482
	Mn		3.8 ± 0.3	3.8 ± 0.6	4.0 ± 0.4	3.8 ± 0.3	0.08	0.908	0.590	0.915
	Zn		83.6 ± 18.6	72.5 ± 18.2	87.4 ± 17.7	71.4 ± 12.6	3.71	0.196	0.226	0.137
Kidney [mg/kg]	Cu	mg/kg	4.1 ± 0.6	4.6 ± 0.8	4.3 ± 0.8	4.2 ± 0.3	0.13	0.641	0.578	0.400
	Fe		27.2 ± 2.5	32.2 ± 4.3	29.9 ± 2.3	28.9 ± 3.2	0.70	0.101	0.307	0.075
	Mn		1.7 ± 0.2	1.9 ± 0.1	1.8 ± 0.2	1.9 ± 0.1	0.03	0.062	0.071	**0.027**
	Zn		23.4 ± 3.0	26.9 ± 3.3	25.2 ± 1.7	24.8 ± 1.4	0.56	0.150	0.230	0.117
Serum [mg/L]	Cu	mg/L	1.9 ± 0.2	2.0 ± 0.2	2.1 ± 0.3	2.0 ± 0.2	0.04	0.677	0.432	0.864
	Fe		1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	0.04	0.851	0.767	0.892
	Zn		1.1 ± 0.0	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	0.02	0.158	0.113	0.742

[1]Group = The aimed amount of Cu in a complete feed. [2]Cu source = $CuSO_4$: Cu(II) sulphate pentahydrate; Cu-Gly: glycine Cu chelate hydrate. [3]Three outliers were identified and removed from the data set to ensure normal distribution. [4]Trace element concentrations in tissues refer to mg/kg or L fresh matter.

Table 4. Mean concentration (±SD) of copper (Cu) in faeces of pigs fed experimental diets with three different levels and two different sources of Cu during 14 weeks of fattening.

Group[1], Cu dosage	Cu Source[2]	mg/kg [DM][3]
Cu-low, 5 mg/kg	$CuSO_4$	$56^a \pm 5$
Cu-medium, 15 mg/kg	$CuSO_4$	$82^a \pm 13$
Cu-medium, 15 mg/kg	Cu-Gly	$147^b \pm 57$
Cu-high, 25 mg/kg	$CuSO^4$	$192^b \pm 88$
	SEM	10.2
p-Value	Group	**< 0.0001**
	Source	**0.018**
	Dosage	**< 0.0001**
	Time	0.163

[1]Group = The aimed amount of Cu in a complete feed. [2]Cu source = $CuSO_4$: Cu(II) sulphate pentahydrate; Cu-Gly: glycine Cu chelate hydrate. [3]Two outliers were identified and removed from the data set to ensure normal distribution. [a-b]Values with different superscripts differ significantly at $p < 0.05$.

3.2. Trace element concentration in the analysed tissues

Tissue trace element results, represented as mg/kg or L fresh matter, are summarised in Table 3. Copper concentrations in liver, muscle, kidney, and blood serum were not influenced by Cu concentration or source in feed. The Cu levels in the tissues examined were in the following order: liver > kidney > blood serum > muscle. Individual Cu concentrations in tissues ranged from 6.3 to 12.1 mg/kg (liver), 3.2 to 6.0 mg/kg (kidney), 1.8 to 2.8 mg/L (blood serum) and 0.4 to 1.2 mg/kg (muscle) across all groups.

No effects were observed for Fe and Zn in the analysed tissues (liver, muscle, kidney and blood serum) with respect to the fixed variables (feeding group, Cu source, and Cu dosage). The Mn content in the kidney showed different dependencies with a trend for the feeding group and the Cu source, as well as a significant influence with increasing Cu content in the feed. The significant dosage effect can be attributed to the influencing effect of the random factor breed. A comparison between the breeds across all groups showed a tendency towards higher Mn levels in the kidney of Saddleback pigs (Landrace hybrid: 1.75 ± 0.2 mg/kg; Saddleback hybrid: 1.88 ± 0.1 mg/kg; $p = 0.083$). No further differences were found with regard to the trace element contents in the individual matrices.

There was no correlation between Cu and the other trace elements regarding the different matrices of liver, kidney, muscle and blood serum (data not shown).

3.3. Excretion of copper via faeces

The Cu content of the faecal samples, represented as mg/kg dry matter (DM), is presented in Table 4. Copper concentrations in faeces differed significantly regarding Cu dosage and source. Faecal Cu concentration increased proportionally with the Cu concentration in feed ($R^2 = 0.9776$). In the Cu-high group, Cu excretion was 3.4 times higher than in the Cu-low group. At a dietary Cu level of 15 mg/kg complete feed, faecal excretion was significantly higher when the organic source was used. The duration of feeding had no effect on Cu excretion. Individual Cu concentrations in faeces ranged from 50 to 63 mg/kg (Cu-low), 56 to 108 mg/kg (Cu-medium $CuSO_4$), 72 to 392 (Cu-medium Cu-Gly) and 54 to 224 mg/kg (Cu-high).

4. Discussion

According to the GfE, the Cu supply recommendation for fattening pigs is 4–5 mg/kg of complete feed (GfE Gesellschaft für Ernährungsphysiologie 2006). The targeted Cu content in the feed of the Cu-low group was about twice as high, at 9.3 mg/kg (Table 1). Therefore, we can conclude that this concentration of Cu in the feed was sufficient to achieve good growth performance in healthy fattening pigs. Our performance data can be compared well with the literature, according to which Landrace pigs are characterised by better performance compared to Saddleback pigs, accompanied by higher daily weight gains (Saddleback pigs: 784 g; Landrace pig: 993 g) and better feed conversion (Saddleback pigs: 3.25; Landrace pig: 2.69) (Paulke 2012). The fact that the performance data for the Saddleback pigs in our trial are even slightly better is mostly due to the crossbreeding with Pietrain. Higher Cu levels in the feed (15–25 mg/kg) did not improve the tested performance parameters. This is in line with other studies that showed no differences in key performance parameters in fattening pigs when Cu supplementation ranged from 5 up to 25 mg/kg of complete feed (Wen et al. 2022; Gourlez et al. 2024). Even if experiments are usually carried out under optimal conditions (e. g. hygiene, space), reductions in the use of Cu as a feed additive are also possible in commercial pig farming through targeted optimisations in management. However, reducing the Cu supplementation in the feed for fattening pigs is easier to implement without fearing a decline in health and performance than it is with piglet rearing, where much higher levels are permitted, with up to 150 mg/kg complete feed, due to the pharmacological effects of Cu. But studies show, that the comparison of health and performance-related parameters were without differences when the concentrations of Cu (and Zn) in the diets of young pigs are reduced (Ding et al. 2021; Gourlez et al. 2024) respectively that high Cu intakes are not crucial for the growth-promoting effect (van Baal et al. 2024). An improvement in performance parameters with high Cu doses is therefore not linked to the endogenous Cu status, and Cu excretion through faeces is subject to homoeostatic regulation. Wen et al. (2022) showed, that the performance of fattening pigs was reduced when no Cu was additionally added to the feed, though the native Cu content was even higher than in the present experiment (7.2 mg/kg versus 5.2 mg/kg) and both native contents would correspond to the GfE recommendation for covering requirements. The native Cu content of a feedingstuff depends on its composition. For instance, barley and wheat have lower Cu contents than soy and maize. Additionally, the availability of Cu may be influenced by different binding forms with nutritional ingredients and by the presence of Fe, Zn, Ca and phytic acid in the feed (Espinosa and Stein 2021). In addition to Cu, the trace element Zn is also added to pig feed, often in concentrations that exceed the animals' requirements by far, but a reduction would also be possible, as shown before (Boerboom et al. 2022; Gourlez et al. 2024). The present study did not focus on different Zn concentrations in the feed, although a high variation in the rations (range: 78–131 mg Zn/kg complete feed) was observed despite the same target incorporation rate (50 mg/kg complete feed, Table 1). Since Fe and Mn also show some fluctuations despite the same target dosage, we suspect a segregation of the trace elements. With regard to the trace element contents in the matrices analysed (Table 3), the segregation, which could be associated with daily fluctuating element contents via the diet, obviously had no influence. Nevertheless, it should be noted that trace elements are generally very well homeostatically regulated, we only sampled on one experimental day (slaughter) and fattening pigs most likely react much less sensitively to different trace element contents in the feed than, for example, piglets would. Mixing trace elements as precisely as possible and choosing a pelleted feed could reduce the risk of segregation in similar feeding trials.

Differences in Cu concentrations in the liver were not anticipated given the study design. As noted in the EFSA review on the use of Cu in animal nutrition, significant increases in liver Cu levels in pigs only become measurable at feed concentrations of 100–150 mg Cu/kg complete feed (EFSA 2016). This is supported by comparable Cu concentrations observed in the livers of our pigs (6.3–12.1 mg/kg), independent of the feeding group. We assume that statistically relevant increases in Cu levels in muscle and kidney tissues would require much higher Cu supplementation, too. There are only a few studies on the transfer of Cu to edible tissues in fattening pigs. Among the current studies, only that of Wen et al. (2022) could be found, which examined the Cu content in the kidney and muscle tissue depending on the Cu content in the feed. However, the Cu concentrations are much higher (muscle: up to 2 mg/kg, kidney: up to 30 mg/kg), so that the results are difficult to compare with ours. In blood, Cu concentrations are generally well-regulated by homoeostatic mechanisms, and even studies involving a higher Cu concentration in feed showed no significant effects (van Baal et al. 2024). The unaffected Cu concentrations in liver, muscle, kidney and blood serum, combined with the observed increase in faecal Cu concentrations as feed Cu levels increased, indicate that Cu homoeostasis at the dietary levels used in this study remained balanced in pigs. Additionally, the increase in Cu excretion through faeces can be considered as evidence of meeting the Cu requirement. Higher levels of Cu in the feed are also reflected in higher levels in the bristles (Gümpel-Schlüter et al. 2024), but these were not examined in our study.

Our study clearly shows that compliance with the maximum Cu concentration in feed (25 mg/kg; EU 2018) ensures that the maximum residue levels (MRL) for Cu in meat (5 mg/kg), liver (30 mg/kg), and kidney (30 mg/kg), as specified in Regulation (EC) No 396/2005 (EC 2005), are not exceeded. Regulation (EC) No 396/2005 establishes the MRL of pesticides in or on food and feed of plant and animal origin. However, in the case of Cu, residues in foods of animal origin partly originate from animal nutrition, too. In the German Total Diet Study, which was aimed to determine average concentration of (undesirable) substances in human nutrition, Cu concentrations were measured in a variety of foods (Kolbaum et al. 2023). The authors reported exceedances of the maximum permissible Cu content in the livers of sheep and cattle, but not in the livers of pigs. The highest Cu concentration found in pig liver was 18.8 mg/kg, which was approximately 1.5 times higher than the highest value measured in the present study (12.1 mg/kg; Cu-medium group of Cu-Gly), but still well below the MRL, too. In contrast, within the NRCP, exceedances of the MRL of Cu in the liver of pigs were repeatedly reported, e. g. with a maximal concentration of 68.9 mg Cu/kg pig liver in 2021 (NRCP 2021). In the NRCP, among other food samples, liver is tested for residues of pharmacologically active substances in a standardised manner throughout the EU. This also takes into account imported food from third countries. Since non-EU countries may have different legal maximum levels for the use of Cu in animal feed and plant protection, it is likely that pig livers exceeding the MRL of Cu are imported from third countries.

To date, many studies have investigated the influence and bioavailability of organic and inorganic forms of Cu in animal nutrition (e.g. Zhao et al. 2014; Kim et al. 2022). Organic Cu preparations are often characterised by a higher bioavailability (Wu et al. 2024). In the present study, Cu excretion in faeces was significantly higher in pigs supplemented with Cu-Gly, although no differences in animal performance or Cu concentrations in tissues were observed in contrast to $CuSO_4$. The higher excretion may indicate that a lower amount of the organic Cu feed additive would be sufficient to meet the animals' requirements and achieve similar performance effects. To confirm this hypothesis and to investigate the bioavailability of organic and inorganic Cu preparations in more detail, a balance study would be required (Brugger et al. 2022). Nevertheless, other studies have also shown differences in Cu excretion. At a dosage of 20 mg Cu/kg complete feed, excretion was significantly higher when an amino acid-bound Cu source was used compared to $CuSO_4$ (Wen et al. 2022).

In terms of environmental protection, reducing the entry of heavy metals into manure is playing an increasingly important role. Animal nutrition can make a contribution to this. In particular, the reduction of feed additives containing Cu and Zn is a topical issue in this context. Adapted feed composition to meet individual animal requirements, as recommended by scientific societies (e.g. GfE), could support this goal. As part of the authorisation process for feed additives, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) also assesses environmental safety. Studies of 840 European soil samples (topsoil) show that the Cu concentration ranges from 0.81 to 256 mg/kg (Salminen et al. 2005), whereby the 90^{th} percentile with a value of 35 mg Cu/kg soil is regarded as the natural background concentration (EFSA 2023). As the expected calculated Cu input into the soil via the livestock manure is lower than the current background contamination, EFSA concludes that the use of Cu containing feed additives for terrestrial animal species is safe for the environment, taking into account compliance with the maximum permitted levels (EFSA 2023). A few years ago, a comprehensive assessment of the input and output of Cu in pig farming operations indicated that that Cu outputs exceed inputs. The authors conclude, that other sources beyond Cu-containing feedingstuff, such as carriers for medicinal substances, mineral bedding materials, and manure additives, also contribute to the Cu content of manure (Schultheiß et al. 2004; Zethner et al. 2007). Koch et al. (2021) reported increased Cu levels in certain bedding materials such as biochar and disinfectant powder. Similarly, Giergiel et al. (2019) documented cases of Cu poisoning in poultry from bedding material, identifying the risks associated with such sources. These findings highlight the need to revise regulations and develop alternative strategies to reduce Cu input from all possible sources in livestock farming.

5. Conclusion

A Cu concentration of about 9.3 mg/kg of complete feed was sufficient enough to achieve good growth performance in healthy fattening pigs. Higher Cu levels in the feed (15–25 mg/kg), regardless of the source, did not provide any benefit in terms of animal performance, but also had no effect on Cu accumulation in edible tissue. A higher level of Cu in the ration was associated with an increase in faecal Cu excretion, indicating that Cu requirements were met and homoeostasis remains balanced in the fattening pigs. In terms of meeting the requirements while reducing environmental impact, organic Cu supplements may offer a benefit but needs further confirmation. To minimise the potential environmental impacts of Cu from manure, the Cu content in the feed should be adjusted more closely to the real requirements of pigs.

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Ethics approval

Ethical approval was obtained from the Regional Office for Health and Social Affairs Berlin (LaGeSo, Berlin, Germany; Registration number: StN 001/22).

Data availability statement

The data supporting the results and analyses presented in this paper are available from the authors upon reasonable request.

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