

Article

Trace Mineral Solubility and Digestibility in the Small Intestine of Piglets Are Affected by Zinc and Fibre Sources

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Abstract: Findings that Zn and fibre source affected the nutrient apparent total tract digestibility (ATTD), made us hypothesize that interactions could occur affecting the apparent digestibility of Zn and trace elements (TEs) interacting with Zn in the digestive tract. Therefore, the study investigated the effects of Zn and fibre sources on the apparent digestibility and solubility of TEs (Zn, Cu, Fe, Mn) and pH in the small intestinal segments of 40-days-old piglets. In vitro solubility of TEs was estimated using a simulated digestion assay. Feed supplementation with potato fibre (PF) affected the ATTD of all TEs and dry matter as well as mineral solubility in the ileum and/or jejunum without any effect on pH in the small intestine. Intake of PF enhanced Zn and Cu absorption ($p < 0.01$), but significantly decreased ATTD of Fe and Mn ($p < 0.001$). Diet supplementation with Zn glycinate decreased Zn absorption in the gut ($p < 0.01$) and affected the solubility of other TEs in the different digestion phases. Although in vitro solubility of TEs does not provide a good prediction of mineral bioaccessibility, using a combination of in vitro and in vivo methods can enable prediction of the trace mineral absorption.

Keywords: trace minerals; fibre; zinc; apparent digestibility; bioaccessibility; piglets



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1. Introduction

The bioavailability of trace elements (TEs) depends on their bioaccessibility, absorption, retention, and role in animals [1,2]. Whereas bioavailability of TEs in vivo is determined by the mineral fraction, which is eventually absorbed into the systemic circulation and utilised by animals, in vitro studies define TE bioaccessibility as the fraction of minerals that is soluble in the gastrointestinal tract (GIT) and available for absorption [2,3]. Trace elements are essential for living animals to maintain their health and performance, but the generally high presence of phytate in cereal-based feedstuffs decreases the absorption of minerals from the gut and their bioavailability [4]. In the digestive tract, divalent minerals chelate with phytic acid to form soluble and/or insoluble mineral-phytate complexes. These complexes are resistant to hydrolysis by phytase activity, and for this reason, the absorption and bioavailability of trace elements are decreased [5]. In addition, pH is an important factor influencing phytate and mineral complex solubility. During passage through the small intestine, at intestinal pH (pH 6.5) the mineral complexes become insoluble and are precipitated to decrease mineral absorption due to de novo complexation [5,6], whereby the trace minerals most affected by phytate are Zn and Fe.

Zinc as a component of many metalloenzymes performs multifarious physiological roles and is involved in almost every metabolic pathway of the body [7]. Zn compounds positively affected the growth performance, intestinal morphology, and regulation of the

gut microbiota; therefore, a therapeutic dose of ZnO is used to decrease the post-weaning diarrhoea frequency in piglets [8,9]. Due to a total ban on the therapeutic use of ZnO that will be introduced from June 2022 in the European Union [10], the evaluation of different Zn sources and Zn lower doses in pig nutrition is very required and challenging. Although the allowed maximum of 150 mg Zn/kg in complete feed in the EU is an acceptable Zn level to maximize the pig performance and effectively counteract diarrhoea [11], it is necessary to improve Zn efficiency and absorption by feed composition in order to maintain animal health, welfare and productivity together with the substantial reduction of environmental pollution from animal husbandry.

Because of the different chemical properties of various Zn feed additives, solubility and interaction of TEs within the GIT may vary. Zinc sulphate together with zinc oxide are the most commonly used Zn source in pig nutrition [12]. ZnSO₄ has been frequently used as a standard to compare the bioavailability of Zn from different sources [13,14], but this soluble source of Zn could more readily interact with phytates and other ligands in the GIT, resulting in lower Zn absorption [15–17]. On the other hand, different chemical structures and properties of Zn organic chelates, more stable Zn complexes, could protect Zn from interaction with other minerals and feed compounds in the gut [2,18], thus making Zn more absorbable [13,19]. Moreover, some Zn organic sources could be absorbed from the intestine in an intact form [18]. Although phytate seems to be the most effective inhibitor of mineral absorption [15], other components such as dietary fibre, lignin, and polyphenolic compounds may bind minerals and affect their bioavailability as well [15,20]. Generally, fibre and compounds associated with fibre in cereal products reduced the mineral absorption in animals [21].

There are two general mechanisms for the inhibition of Zn absorption in the GIT, chelation precipitation (formation of insoluble complexes with phytic acid, dietary fibre, tannins) and competitive inhibition between minerals [22,23]. Feed supplementation with Zn and fibre from different sources (inorganic or organic Zn, and cellulose or potato fibre) could influence mineral absorption during the passage of feed through the small intestine. Our previous results indicated positive effects of potato fibre (PF) supplementation on nutrient digestibility [24]; therefore, we hypothesized that both dietary sources could also affect the apparent digestibility of TEs due to changes in pH and mineral solubility in the GIT. We focused on solubility and absorption of Zn and other interfering TEs in the small intestine as their main absorption site. In this study, we investigated the effect of Zn and fibre sources on the apparent digestibility of TEs (Zn, Cu, Fe, Mn) in the total digestive tract and in the small intestinal segments of piglets as well as pH in the gut. Moreover, we investigated in vitro solubility of TEs from the dietary treatments in a simulated digestion assay, and TE in situ solubility from the small intestinal digesta (duodenum, jejunum ileum) of piglets as a potential indicator of mineral bioaccessibility. Our in vitro experiment was designed to compare the influence of both dietary sources (Zn sources: ZnSO₄ or ZnGly, fibre sources: cellulose or potato fibre) on mineral solubility in the whole GIT using a simulated three-step digestion assay (gastric, small and large intestinal phases).

2. Materials and Methods

All experimental protocols involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and Animal Research: Reporting In Vivo Experiments (ARRIVE guidelines). All methods and procedures reported herein were carried out in line with European Union Directive 2010/63/EU for animal experiments, and the experimental protocol was approved by the Local Animal Experimentation Ethics Committee (resolution number WAW2_21/2016, Warsaw University of Life Sciences-SGGW, Warsaw, Poland) and Polish Law on Animal Protection.

2.1. Experimental Design

2.1.1. Experiment In Vivo

Animals and Diets

A total of twenty-four Danbred $\times$ Duroc barrows (body weight 10.8 ± 0.8 kg) at the age of 40 days were randomly divided into four dietary treatment groups, each fed a cereal-based diet (Table 1) supplemented with Zn and fibre from different sources during a four-week feeding period. The experimental diets consisted of cellulose (Lonocel, Cargill Poland Ltd., Kiszkowo, Poland) and zinc sulphate monohydrate for control treatment (C), cellulose and zinc glycinate (Glycinoplex-Zn, Phytobiotics Futterzusatzstoffe GmbH, Eltville, Germany) for ZnGly treatment, and potato fibre (Potex, Lyckeby Starch, Kristianstad, Sweden) with ZnSO₄ for PF treatment or potato fibre with zinc glycinate for PF + ZnGly treatment. All experimental diets were prepared to contain a total of 150 mg Zn/kg and up to 50 g/kg of total crude fibre content in the complete diet (as-fed basis) from different Zn and fibre sources. Chemical composition of both dietary fibre preparations was published in our previous study [24]. The analyzed total content of Zn and crude fibre (CF) in the experimental diets were 141.9 mg Zn/kg and 42 g/kg of CF for C treatment, 142.2 mg Zn/kg and 40 g/kg of CF for the ZnGly, 141.1 mg Zn/kg and 40 g/kg of CF for PF, and 142.2 mg Zn/kg and 40 g/kg of CF for PF + ZnGly treatments. The ingredients and chemical composition of the cereal-based diets are shown in Table 1.

Table 1. Ingredients and chemical composition of the cereal-based diets.

Ingredients (g/kg)	Diets ¹		Analysed Composition (g/kg)	Diets ¹	
	C	PF		C	PF
Barley	200	200	Dry matter	889	890
Wheat	450	450	Crude ash	47	49
Soybean meal	170	170	Crude protein	182	183
Yellow lupin	60	60	Crude fat	42	41
Rapeseed oil	25.5	25.5	Crude fibre	41	40
Corn starch	33	-	Sugars	41	39
Cellulose	17	-	Starch	378	379
Potato fibre	-	50	Total phosphorus	5.1	5.08
Mineral-vitamin mix ²	4	4	Phytic phosphorus	1.90	1.68
Monocalcium phosphate	7	7	aNDFom	108	112
Calcium carbonate	19	19	ADFom	55	56
Sodium chloride	3	3	Lignin (sa)	11	10
L-Lysine	6	6	Cellulose	43	47
DL-Methionine	2	2	Hemicellulose	53	55
L-Threonine	3	3	Zn (mg/kg)	142	141
L-Tryptophan	0.5	0.5	Mn (mg/kg)	99	94
			Cu (mg/kg)	125	132
			Fe (mg/kg)	315	300
			Gross energy (MJ/kg)	16.8	16.6

¹ C—the diets containing cellulose fibre, PF—the diets containing potato fibre. ² Provided per kg of diet: 14,680 IU vitamin A, 1835 IU vitamin D3, 138 mg vitamin E, 4.59 mg vitamin K3, 2.94 mg vitamin B1, 7.34 mg vitamin B2, 4.40 mg vitamin B6, 44.0 µg vitamin B12, 36.7 mg nicotinic acid, 16.0 mg calcium D-pantothenate, 1.47 mg folic acid, 147 µg biotin, 228 mg Fe, 73.4 mg Mn, 156 mg Cu, 631 µg I, 411 µg Se.

Animal Performance

All piglets were housed in individual pens with free access to feed and fresh potable water. They were kept in a 12 h light/12 h dark regimen with housing temperature maintained at 25 °C. Health monitoring of all piglets was performed daily. Individual performance of feed intake and body weight were measured weekly. At the end of the 28-day feeding period, final body weight and total feed intake were used to calculate feed conversion ratio for each dietary treatment.

Apparent Digestibility

Ten days before the end of the experiment, Cr_2O_3 was included in the diets (3 g/kg diet) as an ingestible marker for estimation of the apparent digestibility of TEs. After five days of adaptation, excreta samples were collected for five days and pooled from each animal and stored at -20°C until freeze-drying. At the end of the feeding period, the piglets were stunned with an electric shock and exsanguinated, followed by the collection of the small intestinal content. pH values in the digesta of duodenum, jejunum, and ileum were measured using a digital pH meter. The collected digesta samples from the duodenum, the middle part of the jejunum, and ileum and pooled excreta samples were freeze-dried, ground, and stored until further analysis. Dry matter (DM) total apparent digestibility was estimated from collected pooled excreta samples. The apparent digestibility of TEs and DM was calculated using the following formula (1):

$$\text{Apparent digestibility of TEs/DM, \%} = 100 - [(\text{Cr}_2\text{O}_3 \text{ in diet} \times \text{TEs/DM in digesta and/or faeces}) / (\text{Cr}_2\text{O}_3 \text{ in digesta and/or faeces} \times \text{TEs/DM in diet}) \times 100] \quad (1)$$

2.1.2. Experiment In Situ

After slaughtering the piglets from each dietary treatment group (in vivo experiment), we collected the digesta from their duodenum, jejunum, and ileum to investigate the effects of Zn and fibre source on in situ solubility of Zn, Cu, Fe, and Mn in the small intestine. Measuring the solubility of these trace elements (TEs) from the digesta samples was done using the technique of centrifugation and a method based on Kleinman et al. [25]. Briefly, 0.5 g of digesta sample from each small intestinal segment was put into a bottle together with 25 mL ultra-pure water (EASypure II UV/UF, Werner Reinst-wassersysteme, Leverkusen, Germany). Then, the samples were shaken at 180 rpm for 60 min. After centrifugation at 6000 rpm for 10 min, the supernatants were filtered through filter papers (Whatman 541), and the soluble content of TEs in the supernatants was analysed directly by atomic absorption spectrophotometry (AAS). The in situ soluble content of TEs was calculated according to the following Equation (2):

$$\text{In situ soluble TEs, \%} = (\text{soluble TEs in digesta supernatant} / \text{total TEs in the diet}) \times 100 \quad (2)$$

2.1.3. Experiment In Vitro

In Vitro Solubility of Zn Sources

We estimated the effect of pH of buffers simulating the GIT environment on in vitro Zn solubility from both Zn supplements (zinc sulphate, zinc glycinate) at three concentrations 20, 100, and 150 mg Zn/L of buffer. Aliquot amounts of ZnSO_4 and ZnGly were placed into bottles with magnetic stirring and dissolved in 0.2 M glycine-HCl buffer at pH 2.0 to simulate gastric digestion and in 0.2 M sodium acetate simulating pH in the small intestine at pH 4.5 and 6.5 [26]. The mixtures were incubated in a shaking bath at 39°C for 4 h to simulate digestion, and filtered through 541 Whatman papers for Zn analysis using AAS. All incubations were done in duplicate, and in vitro solubility of ZnSO_4 and ZnGly infiltrates was calculated according to Equation (3):

$$\text{In vitro solubility of Zn source, \%} = (\text{soluble Zn content} / \text{total Zn content}) \times 100 \quad (3)$$

In Vitro Simulated Solubility of TEs

In vitro simulated solubility of Zn, Cu, Fe, and Mn from four experimental diets differing in Zn and fibre source (see detailed description of in vivo study, Table 1) were estimated using a three-step in vitro simulated digestion assay applying the method of Boisen and Fernández [27]. This assay procedure investigated the solubility of the trace elements in the simulated stomach, small and large intestine digestion.

From each experimental diet (Table 1), nine dried and ground sub-samples (500 ± 0.1 mg) were weighed into pre-weighed bottles, then 25 mL of 0.1 M phosphate buffer (pH 6.0) and 10 mL of 0.2 M HCl were added, and the mixture was stirred with a magnetic bar. To mimic the gastric phase (GP), the pH of the mixture was adjusted to pH 2.0 with 1 M HCl and 1 mL of freshly-prepared pepsin solution (25 mg of pepsin/mL; P7000, Sigma Aldrich, St. Louis, MO, USA). Then, 0.5 mL chloramphenicol (0.5 g/100 mL ethanol, C-0378, Sigma Aldrich, St. Louis, MO, USA) was added to the mixture before incubating at 39 °C for 60 min in a water bath with shaking (150 rpm).

After the gastric phase incubation, the bottles were taken out of the water bath, and to mimic the small intestinal phase (SIP), 10 mL of 0.2 M phosphate buffer (pH 6.8) and 5 mL of NaOH (0.6 M) were added to the mixture and magnetic stirring was resumed. After pH was adjusted to 6.8 (with 1 M HCl or 1 M NaOH), 1 mL of pancreatin solution (100 mg of pancreatin/mL; P1750, Sigma Aldrich, St. Louis, MO, USA) was added, and then closed bottles were incubated for 4 h at 39 °C in a water bath.

The small intestine phase incubation was followed by the last process of simulated digestion phase in the large intestine (LIP) with 10 mL EDTA (0.2 M) added and pH adjusted to 4.8 with 30% glacial acetic acid. The mixture was carefully stirred with 0.5 mL of Viscozyme multi-enzyme complex (Viscozyme L V2010, Sigma-Aldrich, St. Louis, MO, USA) to simulate microbial fermentation in the large intestine, and incubated at 39 °C for 18 h in a water bath with shaking.

During all incubation periods, the bottles were closed and mixtures were stirred slowly and constantly to simulate feed digestion. At the end of each simulated phase, the pH of the mixtures was measured and 2 mL of samples were collected for centrifuging at $4000 \times g$ for 20 min. The supernatants from each digestion phase were filtered through 0.22 µm filter membranes (Millex GS, Merck Millipore, Tullagreen, County Cork, Carrigtwohill, Ireland) and diluted with ultrapure water for subsequent analysis directly by means of AAS. The solubility of TEs in the filtrates was calculated according to the following Equation (4):

$$\text{In vitro simulated solubility of TEs, \%} = (\text{soluble TEs in the GP, SIP, LIP} / \text{total TEs in the diet}) \times 100, \quad (4)$$

TEs = trace elements, GP = gastric phase, SIP = small intestinal phase, LIP = large intestinal phase.

In Vitro Dry Matter Digestibility and pH

After the large intestinal phase, undigested residues from the incubated bottles were filtered in dried and pre-weighed glass filter crucibles containing Celite (Celite 545, Sigma Aldrich, St. Louis, MO, USA), then washed twice with 10 mL of 96% ethanol and 10 mL of 99.5% acetone, and the crucibles were finally oven-dried for 48 h at 105 °C. After cooling, the crucibles were weighed to estimate in vitro dry matter digestibility (IVDMD) calculated using the following Equation (5):

$$\text{In vitro digestibility of DM, \%} = [\text{DM in diet} - (\text{DM in residue} - \text{DM blank})] / \text{DM in diet} \times 100 \quad (5)$$

At the end of each in vitro simulated phase, pH values in buffers with undigested residues were measured with the pH electrode of a digital pH meter.

2.2. Chemical Analysis

The experimental diets and lyophilized digesta and faeces were ground in a grinding mill to pass a 0.5 mm sieve and analyzed for dry matter according to the Association of Official Analytical Chemists (AOAC) method by drying samples to a constant weight at 105 °C [28]. The same method was used for the estimation of dry matter in vitro and in vivo digestibility [28].

Trace mineral concentrations in the diets, faeces and digesta samples, filtrates, and/or supernatants were analyzed using a double-beam atomic absorption spectrophotometer (AA-7000 Series, Shimadzu Co., Kyoto, Japan). The microwave-assisted digestion method

using closed pressure vessels (MWS 4 Speedwave, Berghof Co., Eningen, Germany) was used for the decomposition of the diets, faeces, and digesta samples [29].

Cr₂O₃ concentrations in faeces and digesta samples were analyzed using the colorimetric method of Kimura and Miller [30]. pH values in the digesta of duodenum, jejunum, and ileum and in in vitro buffers and mixtures were measured with the pH electrode of a digital pH meter (WTW InoLab pH Level 2, Weilheim, Germany).

2.3. Statistical Analysis

Treatment effects on the investigated parameters were analysed according to a completely randomised 2×2 factorial design, with a factorial arrangement of treatments using two-way ANOVA, followed by Tukey's post hoc multiple comparisons test with individual variance computed for pair-wise comparisons, where appropriate. The statistical model included the treatment effects (fibre and zinc source) and their interaction. When the interaction was significant, Fisher's Least Significant Difference test (Fisher's LSD) was applied post hoc to determine significant differences among the means. Two-way ANOVA with the main effect was also included to estimate the effect of pH on in vitro solubility of Zn sources and the effect of the digestion phase on in vitro solubility of TEs. All statistical analyses were performed with the GraphPad Prism statistical software (GraphPad Prism version 9.0.0., GraphPad Software, San Diego, CA, USA). Differences between the mean values of the different dietary treatments were considered statistically significant at $p < 0.05$. Data are expressed as means with standard errors of the difference between column means (SE).

3. Results

3.1. Experiment In Vivo

3.1.1. Animal performance

Feed supplementation with ZnGly increased the daily feed intake and body weight of piglets (ZnGly, PF + ZnGly treatments, $p < 0.05$). The average daily feed intake (mean $\pm$ SE) during the whole feeding period was 0.89 ± 0.05 kg, 0.99 ± 0.08 kg, 0.78 ± 0.02 kg, and 1.00 ± 0.06 kg for C, ZnGly, PF, and PF + ZnGly treatments, respectively. Feed conversion ratios ranging from 1.51 to 1.55 did not differ between the dietary treatments.

3.1.2. Apparent Digestibility

Feed supplementation with ZnGly decreased the apparent total tract digestibility (ATTD) of Zn ($p = 0.01$), but the apparent digestibility of Zn in the duodenum, jejunum, and ileum did not differ between the dietary treatments (Table 2). Intake of both PF diets increased the total apparent digestibility of Zn ($p < 0.01$) compared to the ZnGly treatments as well as DM digestibility in piglets. The highest value DM digestibility was recorded in the PF + ZnGly treatment compared to the C and ZnGly treatments (Table 5).

Intake of the PF diets increased the total apparent digestibility of Cu ($p < 0.01$), but Cu digestibility did not change in the small intestine. Interaction between Zn and fibre sources affected Cu digestibility in the duodenum ($p < 0.05$), with decreased Cu digestibility in the PF treatment compared to the PF + ZnGly treatment ($p < 0.05$).

On the other hand, PF supplementation significantly reduced Fe digestibility from the ileum as well as decreased the ATTD of Fe ($p < 0.001$), while the apparent digestibility of Fe was not changed in the other small intestine segments.

ZnGly in the diet increased duodenal apparent digestibility of Mn ($p < 0.05$). However, the Zn source affected the apparent duodenal digestibility of Mn, Mn absorption did not differ between the dietary treatments in the jejunum and ileum. The effects of both dietary sources and source interaction were observed on ATTD of Mn, whereby negative values in the parameter were found, with the lowest values in the PF + Gly treatment.

No changes in pH were found in any small intestinal segments in our in vivo experiment (Table 5).

Table 2. Apparent digestibility of Zn, Cu, Fe and Mn in the small intestine and total tract of piglets fed diets supplemented with zinc and fibre from different sources.

Apparent Digestibility, %	Dietary Treatment ¹				SE	<i>p</i> -Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
Zinc		Zn digestibility, %						
Total tract	29.08 ^b	21.81 ^a	30.53 ^b	28.56 ^b	1.363	0.012	0.002	0.187
Duodenum	68.47	66.03	64.92	77.06	4.758	0.351	0.441	0.142
Jejunum	60.61	54.36	58.00	57.90	4.597	0.499	0.920	0.512
Ileum	57.31	50.23	55.73	53.95	2.991	0.156	0.724	0.387
Copper		Cu digestibility, %						
Total tract	20.05 ^{ab}	12.94 ^a	23.77 ^{ab}	26.51 ^b	2.965	0.470	0.009	0.114
Duodenum	61.37 ^{AB}	55.27 ^{AB}	52.04 ^A	67.50 ^B	4.982	0.378	0.774	0.043
Jejunum	27.44	21.58	30.07	38.83	5.086	0.806	0.105	0.225
Ileum	31.77	24.75	33.92	35.81	7.37	0.732	0.382	0.553
Iron		Fe digestibility, %						
Total tract	14.42 ^b	16.54 ^b	4.255 ^a	6.064 ^a	2.226	0.391	0.0003	0.946
Duodenum	42.97	37.35	32.37	43.26	8.453	0.426	0.759	0.342
Jejunum	29.79	28.80	23.39	21.76	6.618	0.846	0.330	0.963
Ileum	23.40	30.70	10.86	11.19	5.278	0.480	0.008	0.519
Manganese		Mn digestibility, %						
Total tract	−21.93 ^B	−29.27 ^B	−25.59 ^B	−52.06 ^A	4.051	0.0001	0.0001	0.0001
Duodenum	28.73	43.09	32.61	57.06	9.292	0.0498	0.349	0.5934
Jejunum	16.28	10.73	10.83	20.62	10.03	0.835	0.828	0.456
Ileum	12.85	14.46	14.22	12.55	5.210	0.995	0.959	0.757

¹ C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. ^{a–b} Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 6$). ^{A,B} Means within lines with different superscript letters are significantly different ($p < 0.05$) using by Fisher's LSD post hoc test.

3.2. Experiment in situ

In Situ Soluble TEs

Interaction between Zn and fibre sources affected soluble Zn concentration in digesta of each small intestinal segment (Table 3). Zn solubility in the duodenum and jejunum were not affected by any dietary sources, but the effect of both dietary sources on the soluble concentration of Zn in the ileum was observed. Due to dietary source interaction, significant decreased in situ Zn solubility was observed in the duodenum ($p < 0.01$), but soluble Zn concentration increased in the jejunum ($p < 0.05$) in the PF + Gly treatment compared to the ZnGly and PF treatments. Increased Zn soluble content was observed in the ileal digesta of piglets fed the PF diet in comparison to the other dietary treatments ($p = 0.001$).

Supplementation with PF decreased in situ soluble content of Cu in the jejunum without any significant differences between the treatments.

Zn source affected soluble Fe concentration in the jejunal digesta with significantly decreased soluble content of Fe in the ZnGly treatment to the control treatment (C). Fe solubility in the ileum was affected by both dietary sources ($p < 0.0001$). The highest value was observed in the PF treatment, while the lowest in vitro solubility of Fe was observed in the ZnGly treatment compared to others.

In situ solubility of Mn was affected by both dietary sources in the jejunum and ileum. ZnGly in the diets decreased soluble Mn in the duodenum and ileum, but ZnGly increased Mn solubility in the jejunum. Feed supplementation with PF reduced the soluble content of Mn in the jejunum, on the contrary, increased in vitro solubility of Mn was observed in the ileal digesta in the PF treatment ($p < 0.01$). However, the interaction between both dietary sources affected soluble Mn concentration in the ileal digesta.

Table 3. In situ soluble content of Zn, Cu, Fe and Mn from intestinal digesta after dietary treatments with Zn and fibre from different sources.

In Situ Solubility, %	Dietary Treatment ¹				SE	<i>p</i> -Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
Zinc								
		Soluble Zn, %						
Duodenum	40.11 ^{AB}	52.42 ^B	57.02 ^B	19.30 ^A	9.349	0.1847	0.3929	0.0121
Jejunum	31.83 ^{BC}	25.52 ^{AB}	22.18 ^A	34.40 ^C	2.586	0.2605	0.8823	0.0009
Ileum	19.30 ^A	16.50 ^A	31.92 ^B	19.20 ^A	2.129	0.0007	0.0008	0.0243
Copper								
		Soluble Cu, %						
Duodenum	50.51	54.27	49.86	43.47	3.647	0.7216	0.1267	0.1739
Jejunum	102.8	99.88	75.03	85.33	8.250	0.6088	0.0052	0.3608
Ileum	82.55	83.64	78.99	75.51	3.286	0.7186	0.0832	0.4910
Iron								
		Soluble Fe, %						
Duodenum	15.35	16.25	20.39	14.28	1.955	0.1924	0.4383	0.0827
Jejunum	48.62 ^b	30.48 ^a	45.21 ^{ab}	44.25 ^{ab}	4.483	0.0389	0.2544	0.0621
Ileum	44.94 ^b	29.51 ^a	63.42 ^c	46.25 ^b	3.450	<0.0001	<0.0001	0.8022
Manganese								
		Soluble Mn, %						
Duodenum	89.88 ^b	77.40 ^{ab}	79.00 ^{ab}	44.05 ^a	10.80	0.0366	0.0501	0.3072
Jejunum	39.67 ^b	44.77 ^b	27.73 ^a	39.44 ^b	2.775	0.0032	0.0041	0.2397
Ileum	15.77 ^A	15.58 ^A	25.65 ^B	17.83 ^A	1.698	0.0009	0.0229	0.0295

¹ C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. ^{a-c} Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 6$). ^{A-C} Means within lines with different superscript letters are significantly different ($p < 0.05$) using by Fisher's LSD post hoc test.

3.3. Experiment In Vitro

3.3.1. In Vitro Solubility of Zn Sources

The effects of pH on Zn solubility from both Zn sources in different buffers simulating pH in gastric and small intestine digestion were estimated in vitro (Figure 1). The in vitro solubility of Zn from ZnSO₄ and ZnGly was affected by pH ($p < 0.0001$) and Zn source ($p = 0.003$), with the lowest Zn solubility at pH 6.8 simulating digestion in the lower part of the small intestine (Tukey's post hoc multiple comparisons test with individual variance computed for pair-wise comparisons in Supplementary materials, Figure S1, Table S1). The solubility of ZnSO₄ was found to be significantly higher than Zn solubility from ZnGly (two-way ANOVA with the main effect only, $p < 0.001$, Figure 1).

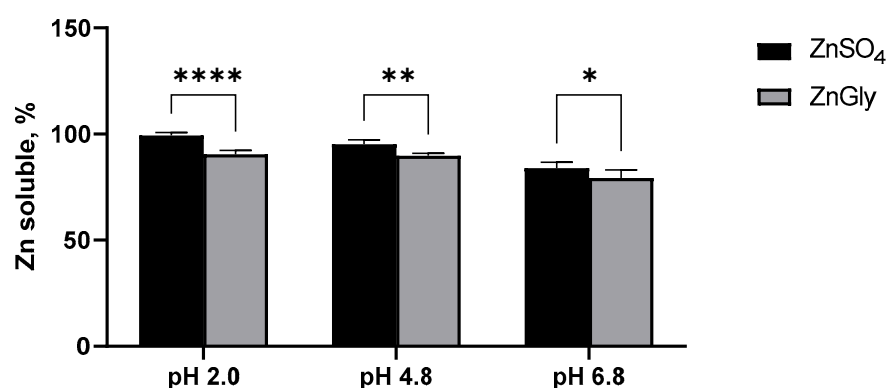


Figure 1. In vitro solubility of Zn from both Zn sources (ZnSO₄, ZnGly) subjected to different buffers simulating gastric digestion in 0.2 M Gly-HCl at pH 2.0, or simulating small intestinal digestion in 0.2 M sodium acetate buffer at pH 4.8 and 6.8; Asterisks represent p -value classification (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

Data were analyzed using two-way ANOVA, followed by the post hoc Tukey's multiple comparisons test, which included the main effect only (pH and Zn source).

3.3.2. In Vitro Simulated Solubility of TEs

In vitro soluble or bioaccessible concentrations of TEs in the experimental diets in each digestion phase are shown in Table 4. Zn, Mn, and Cu were almost totally dissolved in the gastric digestive juice but rapidly decreased solubility of Zn, Mn, and Fe was found in the small intestine phase (SIP). The in vitro solubility of Zn, Fe, and Mn in each experimental diet was observed to be lower in the SIP than in the gastric and large intestinal phases (two-way ANOVA with main effect only, $p < 0.001$); however, Cu solubility did not differ between the simulated digestion phases.

Table 4. In vitro solubility of Zn, Cu, Fe, and Mn from the dietary treatments subjected to three-step in vitro simulated digestion assay.

In Vitro Solubility, %	Dietary Treatment ¹				SE	p-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
In vitro phase		Soluble Zn, %						
Gastric	104.5	109.5	108.0	114.8	6.055	0.3376	0.4766	0.8778
Small intestine	3.229	2.729	1.957	3.771	1.201	0.5893	0.9250	0.3449
Large intestine	119.1	120.1	110.5	124.0	7.032	0.3102	0.7395	0.3804
In vitro phase		Soluble Cu, %						
Gastric	99.37 ^{ab}	110.9 ^b	89.99 ^a	93.63 ^a	3.986	0.0665	0.0022	0.3319
Small intestine	85.52	94.93	79.28	95.40	8.226	0.1293	0.7280	0.6857
Large intestine	95.14 ^{ab}	112.6 ^b	87.99 ^a	100.4 ^{ab}	5.668	0.0119	0.0949	0.6549
In vitro phase		Soluble Fe, %						
Gastric	72.24 ^C	60.64 ^A	66.84 ^B	68.34 ^{BC}	1.558	0.0031	0.4643	0.0002
Small intestine	4.022	2.789	3.644	4.211	0.933	0.7233	0.5796	0.3420
Large intestine	101.4 ^B	78.89 ^A	80.42 ^A	82.05 ^A	4.144	0.0161	0.0381	0.0059
In vitro phase		Soluble Mn, %						
Gastric	90.49 ^a	100.5 ^b	88.30 ^a	96.59 ^{ab}	2.386	0.0006	0.2096	0.7189
Small intestine	11.06	12.10	9.613	12.60	1.586	0.2143	0.7667	0.5434
Large intestine	97.88	103.9	101.3	103.0	3.865	0.3269	0.7445	0.5815

¹ C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. ^{ab} Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison ($n = 9$). ^{A–C} Means within lines with different superscript letters are significantly different ($p < 0.05$) using Fisher's LSD post hoc test.

The in vitro solubility of Zn did not differ between the dietary treatments in each phase of simulated digestion. Intake of PF diets decreased soluble Cu in the gastric phase (GP) and affected solubility of Fe in the large intestinal phase (LIP).

The Zn source influenced in vitro solubility of Cu in LIP, soluble Fe in GP and LIP, and Mn solubility in gastric digestion phases. Supplementation with organic ZnGly increased in vitro solubility of Cu in LIP ($p < 0.05$) and soluble Mn in GP ($p < 0.001$). Zn source also affected the solubility of Fe in GP and LIP; however, the interaction between Zn and fibre sources was observed in both of these digestion phases. Decreased in vitro solubility of Fe in GP was observed in the ZnGly treatment, while the highest soluble Fe concentration in LIP was observed in the control treatment compared to other treatments.

3.3.3. In Vitro Dry Matter Digestibility and pH

Although feed supplementation with PF increased in vivo DM digestibility in piglets, in vitro dry matter digestibility (IVDMD) was not influenced by the dietary treatments (Table 5).

Table 5. Effects of dietary treatments with added Zn and fibre from different sources on pH and dry matter digestibility subjected to in vivo and in vitro digestion assay.

In Vitro, In Vivo Assay	Dietary Treatment				SE	p-Value		
	C	ZnGly	PF	PF + ZnGly		Zn	Fibre	Zn × Fibre
In vitro			pH					
Gastric phase	2.005 ^a	2.010 ^a	2.070 ^{ab}	2.090 ^b	0.016	0.4669	0.0022	0.6589
Small intestinal phase	6.753	6.750	6.753	6.737	0.004	0.0359	0.1232	0.1232
Large intestinal phase	4.773 ^A	4.793 ^B	4.793 ^B	4.773 ^A	0.003	0.9999	0.9999	0.0001
In vivo			pH					
Duodenum digesta	4.385	4.506	4.178	4.858	0.2749	0.1622	0.7946	0.3224
Jejunum digesta	5.505	5.160	5.378	5.505	0.1401	0.4449	0.4449	0.1078
Ileum digesta	7.325	6.818	7.257	6.867	0.2709	0.1135	0.9709	0.8317
Dry matter (DM)			DM digestibility, %					
In vivo assay	83.17 ^a	83.21 ^a	84.67 ^{ab}	85.31 ^b	0.3841	0.3900	<0.0001	0.4502
In vitro assay	74.78	73.93	75.35	76.25	2.404	0.9746	0.5776	0.7392

¹ C—cellulose, PF—potato fibre, ZnGly—zinc chelate with glycine. ^{a–c} Means within a row with different superscript letters are significantly different ($p < 0.05$) as a result of a Tukey's means comparison, means represent 6 replicates in vivo, 9 replicates in vitro (DM digestibility) or 3 replicates in vitro (pH). ^{A–B} Means within lines with different superscript letters are significantly different ($p < 0.05$) using Fisher's LSD post hoc test.

Different effects of Zn and fibre sources on pH values were determined: PF affected pH in the in vitro simulated GP ($p < 0.01$) and Zn source in the SIP ($p < 0.05$). The diets containing PF increased pH in the gastric phase, while ZnGly in the diets slightly decreased pH in the SIP (Table 5). Interaction between both dietary sources affected pH in LIP with increased pH in the Gly and PF treatments.

4. Discussion

Trace elements as essential feed components improve animal health and livestock productivity but feed supplementation with TEs result in a substantial excretion of heavy metals into the environment. Therapeutic use of ZnO or excessive feeding of inorganic Zn to piglets can stimulate resistance in the gut microbiota, and Zn accumulates in faeces and then in manure [31]. Replacement of inorganic feed mineral additives by more bioavailable mineral sources or using enhancers of trace mineral absorption in animal feed could reduce excretion of TEs into the environment and reduce heavy metal emissions from livestock production.

Whole-body homeostasis of Zn, Mn, Cu, and Fe is predominantly regulated by intestinal absorption [2,32]. Absorption of trace elements (TEs) does not only depend on an adequate dietary intake but is also greatly affected by its intestinal availability from the diet. Different chemical species of dietary TEs can interact with other components in the diet before reaching their absorption site and these interactions may considerably modify the metabolically available amount of the trace minerals, mineral metabolism, and their regulation [16]. Sufficient Zn supply from different organic dietary sources of Zn and from inorganic ZnSO₄ showed usually no differences in metabolic utilisation in animals due to Zn homeostasis adapted to intestinal Zn content [33]. However, Zn supplementation with organic ZnGly increased Zn absorption from the GIT of Zn-deficient animals [34,35]. Our results indicated the decreased apparent total tract digestibility (ATTD) of Zn in piglets fed the diet supplemented with organic Zn glycinate compared to other treatments; however, the apparent Zn digestibility did not differ in the small intestine. Higher feed consumption of piglets fed the diets supplemented with ZnGly also increased the ingested amount of Zn that could lead to increased excretion of Zn, and as a result of Zn homeostasis regulating the absorption and excretion of Zn in the GIT [32,36], Zn apparent digestibility decreased in the ZnGly treatment. However, decreased ATTD of Zn was observed in the ZnGly treatment only. It has been known that fractional Zn absorption decreases increasing Zn content in the feed, probably due to a saturation of transport mechanisms [37]. Zn absorption is more efficient in low zinc diets [38] and relates to oral zinc intake [17]; therefore, increased feed

consumption of Zn might decrease Zn uptake by the small intestine and Zn absorption. Moreover, the importance of maintaining Zn balance is endogenous Zn excretion and Zn reutilisation by GIT which directly relates to the absorbed Zn amount [39]. Another reason for the lower ATTD of Zn from ZnGly could be the structure of zinc glycinate. The small molecular size of ZnGly tends to bind in cavities of native fibre [40] resulting in the decrease of apparent Zn digestibility, but it seems that feed supplementation with PF could eliminate the effect. Reduced absorption and retention of Zn from ZnGly were observed in pigs due to the structure and lower average bond strength between the amino acid and Zn in ZnGly [40].

Although it seems that the chemical properties of trace elements are of subsidiary relevance in the absorption process of dietary TEs in the GIT, their chemical speciation is relevant for complex interactions with different chelators to promote or prevent the formation of insoluble mineral complexes [22,34]. Phytates and fibres are the main ligands binding native Zn and other trace element species in the GIT [32,41] resulting in the inhibition of Zn absorption and reabsorption by chelating ingested Zn or secreted endogenous Zn in the intestinal lumen [39]. Regardless of dietary fibre source, fibres may affect mineral absorption due to their mineral binding properties [42,43] and these interactions are important from a nutritional point of view. Cellulose is a poorly soluble and fermentable fibre source that entraps minerals to form large insoluble complexes resulting in decreased mineral bioavailability; however, most of the different fibres can also bind Zn, Fe, Mn, and Cu under *in vitro* conditions [44]. On the other hand, more fermentable PF can enhance the absorption of TEs by lowering pH or the production of organic acids in the GIT [45]. Although no effect of PF on pH in the gut was found in our study, we found the beneficial effect of PF feeding on apparent total tract digestibility of DM, Zn, and Cu, but ATTD of Fe and Mn was considerably decreased. Similarly, fibre source differently affected soluble content of TEs in digesta of the ileum and/or jejunum. Several ligands of different types of dietary fibres are responsible for the metal binding and the binding strength to different metal ions [41]. We can only speculate that the physicochemical properties of PF might differently affect the solubility of TEs in the GIT and their digestibility. Potato starch from potato fibre has a higher viscosity and longer texture in comparison to corn starch [46] used in the C and ZnGly diets, which could influence conditions for mineral absorption in the GIT. Different effects of digestible starch on the apparent intestinal absorption of Fe and Zn were also observed in pigs [47]. Further investigation is needed to elucidate the mechanism of action of potato fibre in mineral absorption and the different effect of PF on the digestibility of TEs.

The amount of trace minerals available for absorption (bioaccessible or soluble amount) depends on the concentration of TEs in the diet, feed composition, and the presence of ligand enhancers or inhibitors of mineral absorption in the GIT [20]. Conditions increasing the solubility of trace minerals or protecting them from interaction with inhibitors in the intestinal lumen are generally beneficial for their absorption and uptake by the apical surface of enterocytes in the small intestine [37]. On the other hand, soluble inorganic species of TEs are more sensible to form insoluble chelate compounds with various chelators at the pH of the gut lumen [48]. In our study, the reduced ATTD of Zn in the ZnGly treatment might be a result of decreased soluble Zn in the ileal digesta of pigs supplemented with ZnGly. Similarly, the highest soluble Zn content observed in the ileum of piglets fed the PF diet could lead to increased ATTD of Zn.

On the other hand, diet supplementation with PF increased the ATTD of Cu in our piglets, while *in vitro* solubility of Cu in the simulated gastric phase as well as *in situ* solubility of Cu in the jejunum decreased. Cu is primarily absorbed through the stomach and small intestine of monogastric animals and gastric acidity promotes the presence of freely-soluble Cu ions [49]. Cu solubility in the gastric phase could predict mineral availability in the rest of the gastrointestinal tract, so decreased solubility of Cu in the simulated *in vitro* gastric phase could be related to the reduced soluble Cu percentage in the jejunum (*in situ*). However, we found interactions between both dietary sources which

might influence the ATTD of Cu in piglets. It has been reported that *in vitro* solubility of Cu might not accurately represent the *in vivo* bioavailability of Cu due to either disassociation or chemical shifts in Cu along the intestinal tract of broilers [50]. In any case, it should be stressed that Zn source had no effects on either Cu solubility or Cu digestibility in the GIT of our piglets, which is an important fact concerning the competitive antagonism for absorption between those two trace minerals.

Feed supplementation with PF negatively influenced the total apparent digestibility of Fe and decreased Fe absorption from the ileum of our piglets. On the other hand, *in situ* solubility of Fe increased in the ileum of piglets fed the PF diet. Solubility of Fe could be affected by changes in intestinal pH in the presence of PF in the diet, but no effect of PF on pH was observed in our study. It seems that even though *in situ* solubility of Fe increased in the ileum, the PF treatment interactions with other feed components along the digestive tract decreased the total digestibility of Fe. Moreover, it has been identified several complexes of Fe which are soluble, but unabsorbable in the gut, using an *in vitro* method measuring Fe solubility to predict Fe bioavailability [51]. *In vitro* and *in situ* solubility of Fe could be affected by Zn source, because ZnGly supplementation decreased Fe solubility in both trials. Similarly, *in vitro* and *in situ* solubility of Mn was affected by both dietary sources, but the effect is inconsistent. It seems that interaction between Zn and fibre sources led to the reduced apparent digestibility of Mn. Therefore, further investigation is needed to fully understand the effects of ZnGly and potato fibre on Fe and Mn absorption and interactions, and also competitive inhibition between the minerals in the GIT.

Another important factor affecting the solubility of mineral complexes in the GIT is the pH level of its various parts. Complexes formed by phytic acid and TEs were soluble under the acidic conditions in the *in vitro* simulated gastric phase, and therefore the highest *in vitro* soluble content of each trace mineral were recorded in this phase. In contrast, the solubility of phytate complexes with Fe increases above pH 4 and they are more insoluble at gastric pH [5], resulting in reduced *in vitro* soluble content of Fe in GP in our study. Moreover, ZnGly in the diet even reduced Fe *in vitro* solubility in GP, but increased soluble Mn. It seems that PF in the diet could affect mineral solubility due to increased pH in simulated *in vitro* GP, but our study design meant that we did not measure pH in the stomachs of our piglets to confirm this assumption *in vivo*. On the other hand, increased Mn digestibility in the duodenum after ZnGly supplementation could probably be a result of higher *in vitro* solubility of Mn in the GP, because mineral solubility in the gastric phase then dictates the availability of those minerals in the rest of the gastrointestinal tract. *In situ* solubility of Mn in the small intestine segments were affected by both dietary sources.

In vitro solubility is one of the methods of assessing the bioaccessible amounts of TEs based on the determination of soluble TEs under simulated physiological conditions [1]. Although bioaccessibility of TEs evaluated by *in vitro* methods is an important step in the assessment of trace mineral bioavailability, more important is the amount of TEs which is absorbed into the systemic circulation, and converted to the physiologically active species [2]. *In vitro* methods are used to identify possible physicochemical properties of feed compounds that could contribute to explain differences in mineral absorption [51]. The prediction of bioavailability of TEs by means of *in vitro* digestion assay is only relative because it does not exactly mimic the *in vivo* digestion due to physiological and environmental factors [52], but the solubility of TEs determined in *in vitro* experiments might represent the bioaccessible amount of TEs in the dietary treatments for pigs. *In vitro* simulated digestion in the SI phase significantly decreased concentrations of soluble TEs except Cu, which could have resulted in no significant differences between the treatments in this phase. At the intestinal pH value used in simulated *in vitro* assay (pH 6.8) or during passage through the small intestine, mineral complexes becoming insoluble and precipitated, thus decreasing mineral absorption due to *de novo* complexation [5,6].

The change in pH and introduction of more enzymes in the simulated small intestinal phase activated a series of reactions leading to complexation, adsorption, and precipitation of TEs, and decreased bioaccessibility of Zn, Mn, and Fe [53]. Phytic acid in the

cereal-based diet chelated Zn, Fe, and Mn (but not Cu), forming insoluble and undigestible complexes [54]. Even though phytic acid binds Cu more strongly than Zn in vitro, complexes with Cu are soluble over a wide pH range and are less precipitated at neutral pH [55]. Moreover, no inhibitory effect of phytic acid on Cu absorption in vivo has been confirmed [56,57] nor on good solubility of Cu-phytate complexes in the intestinal lumen [58,59]. Enzyme phytases originating from plants, microorganisms, and animal tissues catalyse phytate hydrolysis to inorganic phosphate [60]. Endogenous phytases can degrade phytates, and their complexes with TEs in pig nutrition are phytases generated by the small intestinal mucosa, microbial phytase presents mainly in the large intestine, and intrinsic plant phytase derived from certain feedstuffs [61]. We assume that during our in vitro simulated digestion, hydrolysis of phytates and their complexes could take place only through intrinsic plant phytase activity, and therefore the lower in vitro soluble concentrations of TEs in the SI phase was a result of the strong proteolytic activity of proteases in the pancreatin solution at intestinal pH, which broke down the intrinsic plant feed phytases to result in increased formation of insoluble mineral-phytate complexes and their precipitation [62,63]. On the other hand, gut-derived microfloral and mucosal phytases, which are more stable at higher pH in the duodenum than intrinsic plant feed phytases, could have hydrolysed mineral-phytate complexes in the small intestine of our piglets in vivo, so the in situ solubility of TEs and also the apparent digestibility of TEs did not significantly affect the small intestinal segments of the piglets.

Our inconsistent results as well as outcomes of other studies [64] indicated that in vitro mineral solubility to predict bioavailability of TEs from their different sources may sometimes be unsatisfactory and misleading. Discrepancy and poor correlation between soluble content and total apparent digestibility of TEs could be caused by different total and soluble content of TEs in intestinal digesta collected only one-time at the end of the study (in situ soluble TEs) in comparison to the mineral concentration in pooled faeces which were collected continually 5 days (in vivo ATTD). However, it has been concluded that the variability of apparent nutrient digestibility derived after slaughtering was similar to that found with cannulated animals [65]. Moreover, except for dietary factors affecting the mineral bioavailability in vitro and in vivo, there are various physiological factors influencing the trace mineral digestibility in animals. Since the physiological factors including animal species, sex, age, state of growth or physiological and nutritional status of animals are not present in in vitro systems, they cannot affect in vitro digestibility [2].

Various in vitro solubility techniques used for estimating mineral bioaccessibility indicated either a good or poor correlation between in vitro solubility and in vivo bioavailability of Zn and Fe, depending on used in vitro conditions and chemical species of dietary TEs [3,52,65]. As the good indicator of Zn bioavailability from different Zn sources seems to be their solubility in pH 5 and 2 buffers for poultry; however, an inverse relationship was found [14]. On the other hand, the positive relationship was found between Cu bioavailability and Cu solubility in pH 2 buffer [66]. Therefore, in vitro studies for measuring bioaccessibility and/or bioavailability of TEs are not suitable for fully substituted in vivo studies, and should be regarded as screening methods to help us identify dietary factors affecting the absorption of TEs and to study their interactions. There is a need for more validation studies in which the in vivo results are compared to in vitro results.

5. Conclusions

We conclude that feed supplementation with potato fibre can enhance the apparent digestibility of Zn and Cu from the gut of piglets, but it significantly decreased apparent total tract digestibility of Fe and Mn, and Fe apparent digestibility in the ileum. On the other hand, it seems that Zn glycinate is less soluble in the gut than Zn sulphate, resulting in decreased Zn absorption and digestibility in the lower small intestine. Moreover, ZnGly can affect in vitro solubility of other TEs in the different phases of simulated digestion, which could lead to the changed absorbability of TEs. Apparent digestibility of Zn and Cu

was not affected by Zn or fibre source in the separate small intestinal segments, while Mn digestibility in the duodenum increased by ZnGly supplementation.

Although in vitro solubility of TEs did not provide a good prediction of mineral bioaccessibility because of rapidly decreased solubility of Zn, Fe, and Mn in the simulated small intestinal phase, using a combination of the results from in vitro and in vivo experiments might be used for predicting the relative bioavailability of TEs and might help to improve understanding of important factors involved in digestion and absorption processes involving TEs.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture12040517/s1>, Figure S1: In vitro solubility of Zn from both Zn sources (ZnSO₄, ZnGly); Table S1: Individual significant differences between parameters, in vitro solubility of Zn from both Zn sources (ZnSO₄, ZnGly).

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