Effects of chromium supplementation on growth, nutrient digestibility and meat quality of growing pigs

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Abstract

The aim of the study was to evaluate the effects of chromium picolinate (CrPic) on growth performance, nutrient digestibility, and protein and lipid quality of five anatomical parts in growing pigs. The 30-day study was conducted on eight castrated Topigs growing male pigs, with an initial bodyweight of 17.16 ± 0.62 kg. The pigs were assigned to two groups (C, E), housed in individual metabolic cages, and fed on conventional diets with 17.80% crude protein (CP) and 3078 kcal/kg metabolizable energy (ME). The diet of E was supplemented with 200 ppm CrPic. Samples of ingesta and faeces were collected in three balance periods of five days each. At the end of the experiment, blood samples were collected, all pigs were slaughtered, and meat (tenderloin, loin, ham, shoulder, and belly) samples were collected. No significant differences of productive or plasma parameters were noticed. The results of the balance study showed that CrPic did not influence the digestibility of nitrogen, but the digestibility of fat was significantly decreased for group E. The nutritional quality of the collected samples was evaluated for proximate analysis. The tenderloin and ham samples had increased protein concentrations compared with C group. For belly and ham, the fat concentrations decreased significantly. As a result of this observation, amino acids and fatty acid profiles were analysed and a significant improvement were determined for E regarding essential amino acids. The conclusion of the study was that CrPic had positive effects on protein and fat metabolism and the meat had functional food attributes.

Keywords: Amino acids, biochemical profile, chromium picolinate, digestibility, fatty acids, pork
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Introduction

In the living organism, chromium (Cr) is a component of glucose tolerance factor. Its primary role in metabolism is to enhance the glucose uptake by tissues (Pechova & Pavlata, 2007). Chromium can also be supplemented to reduce the negative effects of environmental stress, dietary and hormonal stress (Sahin *et al*., 2002), and immune stress (Heugten & Spears, 1997).

Chromium supplementation to diets for swine may improve specific growth and carcass responses (Jackson *et al*., 2009). It was noticed that the experimental period is a major factor that determines the pig's response to Cr supplementation (Amata, 2013). Several authors (Page *et al*., 1993; Lindemann *et al*., 1995: Mooney & Cromwell, 1995) have reported variable results when the experiments were for the entire growing-finishing period. Sales & Jancík (2011) designed a meta-analysis to describe the effects reported in several studies. According to random effects models, average daily gain, percentage carcass lean, *longissimus* muscle area increased, and 10th-rib fat thickness decreased under dietary Cr supplementation.

Chromium sources used in animal experiments were chromium picolinate (CrPic), chromium nicotinate, chromium chloride and less Cr propionate, but some authors consider that the organic source of Cr is over ten times more bioavailable than inorganic sources (Shelton *et al*., 2003).

There has been limited research on the effect of Cr supplementation on the nutritional quality of various anatomical parts (tenderloin, ham, shoulder, etc). Most published research refers to the *longissimus* muscle area and total carcass lean (Shelton *et al*., 2003), drip loss in chops (Wang *&* Xu, 2004), loin pH, drip and purge loss (Matthews *et al*., 2005), backfat and the iodine value of belly (Jackson *et al*., 2009), carcass lean ratio and carcass fat ratio (Wang *et al*., 2014), and meat colour (Li *et al*., 2013). The concentrations of

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protein and fat are critical attributes of carcass quality (Monziols *et al*., 2006). Protein quality is described by its ability to provide specific concentrations and patterns of amino acids (Millward *et al*., 2008). Saturated and unsaturated fatty acids can provide information about the palatability and rancidity of fat (Hoffman *et al*., 2005).

The purpose of this study was therefore to determine the influence of supplementing Cr as picolinate on growth, plasma metabolites, nitrogen and fat digestibility, and pork quality (amino acids and fatty acid composition of various tissues) in growing pigs.

Material and methods

The experiment was performed in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes and all procedures were described. It was approved by the Ethical Commission of National Research and Development Institute for Biology and Animal Nutrition, Balotesti, Romania.

The experiment was conducted on eight growing castrated hybrid TOPIGS (Large White x Hybrid (Large White x Pietrain) female) × Talent (mainly Duroc) male pigs, aged 57 ± 3 days, under conditions of experiment balance and it ran for 30 days. Throughout the experimental period, the piglets were randomly assigned to two groups (four animals per group), kept in individual metabolic cages (Agrico, Rybarska, Czech Republic) with an area of 0.87 m^2, placed in an experimental hall under controlled environmental conditions (temperature of 24 °C, humidity 50 to 60 %). The piglets were fed their diets daily at 8.00 a.m. *ad libitum*. The amount of feed given to each pig was weighed daily, as well as the leftovers (collected each morning). Water was supplied *ad libitum* via drinking nipples. The pigs had an average initial bodyweight of 17.16 ± 0.6 kg. They received a commercial diet designed for this category of animals, which differed between groups according to the level of Cr^{3+} supplement (Table 1). The source of Cr^{3+} was Cr picolinate (Sigma Aldrich, Germany). It brought the chromium level to 200 µg Cr/kg feed in E group. The productive parameters (average daily gain, feed conversion) were calculated from the records of bodyweights and feed intake.

After five days for accommodation, the nutrient balance was determined during three periods of five days each. During the three periods of balance (5 days/week) samples of excreta (faeces) were collected daily from each animal and average weekly samples were formed. The faeces were collected once a day and stored at about 30 °C. At the end of the collection period, the faeces were weighed and homogenised. Faeces samples were dried at 65 °C in stove BMT model Ecocell Blueline Comfort (Nuremberg, Germany) and ground with a Grindomix GM 200 mill (Retsch, Germany).

The coefficients of apparent absorption of nitrogen and fat were calculated using the data from the chemical analysis on the feeds and faeces, corroborated with the daily records of the intake and excreta, using digestibility equations proposed by Schiemann (1981).

Blood was collected in heparin tubes, centrifuged for 10 minutes at 2500 rpm. The resulting plasma samples were analysed for haematological and biochemical parameters.

At the end of the experiment, after blood sample collection, all pigs were slaughtered in an experimental abattoir. Five cuts (tenderloin, shoulder, loin, ham and belly) were dissected, deboned, external fat removed, frozen at -80 °C and kept until chemical analysis.

The CP of the diet, faeces and muscles was determined using a semiautomatic classical Kjeldahl method using a Tecator Kjeltek auto 1030 analyser (SR EN ISO 5983-2, 2009). The fat was extracted using an improved version of the classical method by continuous extraction in solvent, followed by fat measurement with Soxhlet after solvent removal (SR ISO 6492, 2001).

Gas chromatograph Perkin-Elmer Clarus 500 (Massachusetts, United States), fitted with a flame ionization detector (FID) and capillary separation column with high polar stationary phase TRACE TR-Fame, (Thermo Electron, Massachusetts, United States), dimensions 60m X 0.25 mm X 0.25 µm, was used to determine the fatty acid composition of the meat samples. Each sample was prepared as described previously (Habeanu *et al*., 2011).

HPLC Surveyor Plus Thermo Electron, (Massachusetts, United States) and HyperSil BDS C18 column (Thermo Electron, Massachusetts, United States) dimensions 250mm X 4.6 mm X 5 µm were used to determine the amino acid profiles of the meat samples. Each sample was prepared as described previously (Varzaru *et al*., 2013).

Blood samples were collected from slaughtered pigs at the end of experiment. The blood samples were collected from the jugular vein in tubes with EDTA for hematologic determination (white blood cells (WBC) red blood cells (RBC); haemoglobin (HG); haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC)) and biochemical determinations (total cholesterol and total protein) on a Mindray BC 2800 VET auto haematology analyser (China).

Each pig was considered an experimental unit. All data are expressed as mean value ± standard error of the mean (SEM). The analytical data were compared with analysis of variance (ANOVA), using STATVIEW for Windows (SAS, version 6.0). The differences between mean values in the groups were considered significant at $P < 0.05$.

Table 1 Formulation and chemical composition of compound feeds used for hybrid Topigs piglets

Ingredients (g/Kg as feed bases)	C	E (+ CrPic)
Maize	375.9	375.9
Wheat	260.0	260.0
Rice bran	90.0	90.0
Soybean meal	160.0	160.0
Sunflower meal	40.0	40.0
Corn gluten	30.0	30.0
Calcium carbonate	18.0	18.0
Mono calcium phosphate	9.0	9.0
Salt	2.0	2.0
Methionine	0.5	0.5
Lysine	3.6	3.6
Choline	1.0	1.0
Premix*	10.0	-
Premix**	-	10.0
Calculated nutrients (g/kg feed)		
ME (Kcal / kg)	3078	3078
Crude protein (g)	178.0	178.0
Crude fat (%)	33.9	33.9
Crude fibre (%)	42.0	42.0
Calcium (%)	9.0	9.0
Phosphorus (%)	6.5	6.5

ME-Metabolisable Energy, C-Control, E- Control + chromium picolinate, CrPic- chromium picolinate, *Ingredients per kilogram of diet: 6000 IU of vitamin A, 1500 IU of vitamin D3, 10 IU of vitamin E, 1.5 mg of vitamin K3, 1.0 mg of vitamin B1, 3.0 mg of vitamin B2, 10.0 mg of d-pantothenic acid, 15.0 mg of niacin, 1.0 mg of vitamin B6, 15.0 g of vitamin B12, 250 mg of FeSO4 x 7H2O, 62.8 mg of CuSO4, 197.4 mg ZnSO4 x 7 H2O, 38.8 mg of MnO, 0.7 mg of CoSO4 x 7 H2O, 1.0 mg of KI, 200g of Na2SeO3, and 380.4 mg corn starch as carrier

**Ingredients per kilogram of diet: 6000 IU of vitamin A, 1500 IU of vitamin D3, 10 IU of vitamin E, 1.5 mg of vitamin K3, 1.0 mg of vitamin B1, 3.0 mg of vitamin B2, 10.0 mg of d-pantothenic acid, 15.0 mg of niacin, 1.0 mg of vitamin B6, 15.0 g of vitamin B12, 250 mg of FeSO4 x 7H2O, 62.8 mg of CuSO4, 197.4 mg ZnSO4 x 7 H2O, 38.8 mg of MnO, 0.7 mg of CoSO4 x 7 H2O, 1.0 mg of KI, 200g of Na2SeO3, 200 µg CrPic and 380.4 mg corn starch as carrier

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Results and Discussion

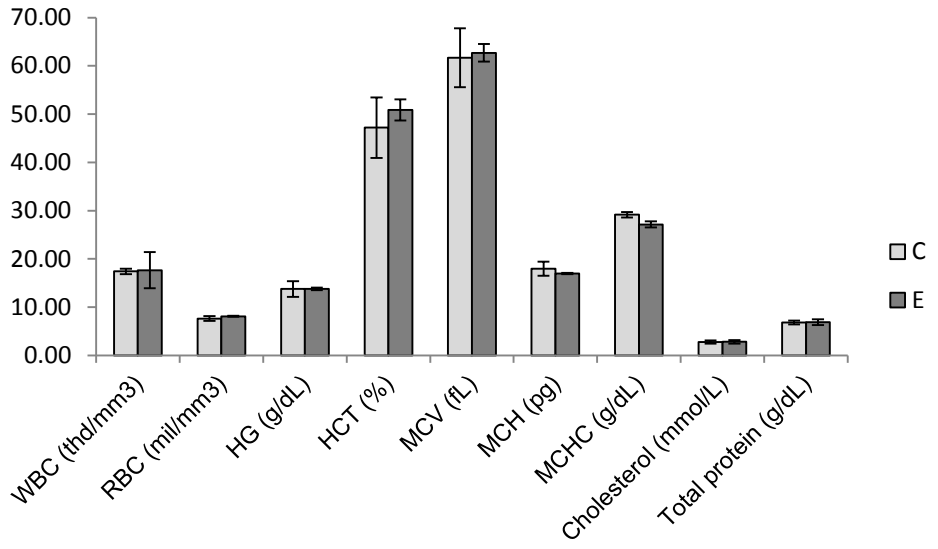
There was no ($P > 0.05$) effect on overall growth performance of pigs fed supplemental CrPic (Table 2). Similar to the present study, Shelton *et al*. (2003), Kornegay *et al*. (1997) and Amoikon *et al*. (1995) reported that supplemental Cr (200 µg/kg Cr picolinate) resulted in no differences between groups for performance parameters when Cr was added to the pigs' diet. Average daily gain was increased, but ADFI and gain : feed were not affected by 200 ppb Cr additions to the diet (Page *et al*., 1993). An improvement in gain : feed ratio but no observed differences for ADG or ADFI were reported by Lindemann *et al*. (1995).

Plasma haematological and biochemistry constituents did not differ ($P > 0.05$) by addition of CrPic (Figure 1).

Table 2 Mean performances (± SEM) of pigs fed two diets differing in chromium picolinate concentration

Items	C	E (+ CrPic)	SEM	P-value*
Initial weight (kg)	17.18	17.14	0.21	0.9341
Final weight (kg)	37.28	36.98	1.23	0.9139
Average daily gain (kg/pig/day)	0.74	0.73	0.05	0.9375
Average daily intake (kg/pig/day)	1.64	1.71	0.10	0.7583
Feed efficiency (kg feed: kg gain)	2.30	2.49	0.26	0.7437

*Means within a row did not differ significantly at *P* <0.05, C-Control, E- Control + chromium picolinate, CrPic- chromium picolinate



WBC: white blood cells, RBC: red blood cells, HG: haemoglobin, HCT: haematocrit, MCH: mean corpuscular haemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration

Figure 1 Haematological and biochemical parameters of pigs fed two diets differing in chromium picolinate concentration at the end of experiment

All pigs from the CrPic supplemented group remained in good health throughout the experimental period. No signs of toxicity were observed. Haematological and biochemical parameters determined in plasma were within normal limits, according Merck manual (2012). The data regarding plasma haematological and biochemical profile agree with other researchers, who showed that Cr supplementation did not affect the plasma metabolites of pigs (Lien *et al*., 2005). Amoikon *et al*. (1995) reported an increase in fasting plasma cholesterol. Other researchers reported a decrease in cholesterol in Cr-supplemented pigs (Page *et al*., 1993).

The results of the balance study (Table 3) showed that 200 ppb Cr(III) produced a slight increase of the apparent absorption coefficients for E group in N balance. Pigs fed diets supplemented with Cr excreted an increased (*P* <0.05) amount of fat but had a decreased (*P* <0.05) apparent absorption coefficient compared with the pigs from the control group.

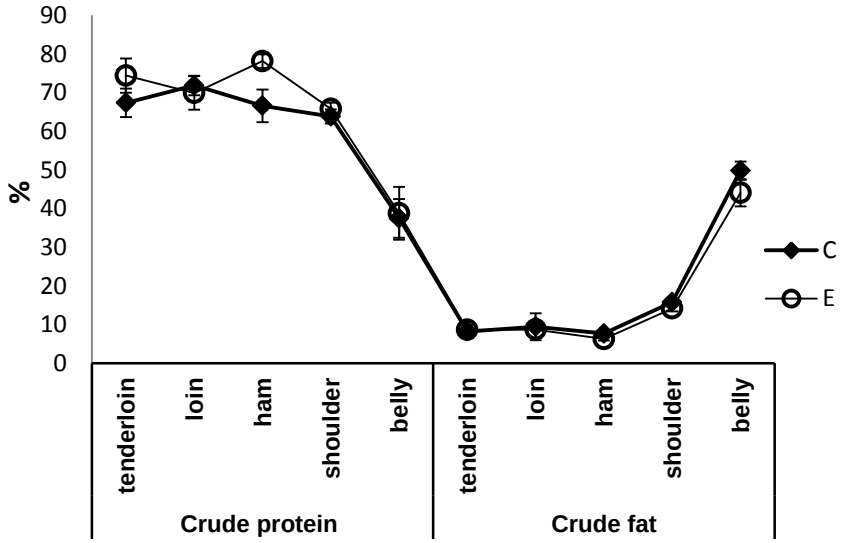
Kornegay *et al*. (1997) showed that 200 ppb Cr as CrPic had a positive influence on the rate of nitrogen (N) absorption and DM digestibility. Lindemann *et al*. (1999) considered that Cr has the potential to increase carcass lean and, in that way, to prevent the negative environmental impact of nitrogen excretion of greater protein intakes. Some researchers have shown that Cr supplementation in monogastric animals had a beneficial influence on lipid metabolism (Wang *et al*., 2009). The results of the current study sustain this opinion, as the apparent digestibility coefficient for fat was decreased compared with the control group.

Table 3 Effect of chromium picolinate supplements on apparent nutrient digestibility

Items	C	E (+ CrPic)	SEM	P-value*
Nitrogen				
Ingested (g/day/pig)	46.27	47.91	2.03	0.6996
Excreted (g/day/pig)	9.34	9.03	0.66	0.8200
Absorbed (g/day/pig)	36.93	39.42	1.47	0.4099
Apparent absorption (%)	80.27	82.41	0.66	0.1067
Fat				
Ingested (g/day/pig)	54.91	59.30	2.41	0.4180
Excreted (g/day/pig)	23.71[b]	30.98[a]	1.87	0.0363
Absorbed (g/day/pig)	31.20	28.32	1.31	0.3175
Apparent absorption (%)	56.96[b]	47.66[a]	1.98	0.0012

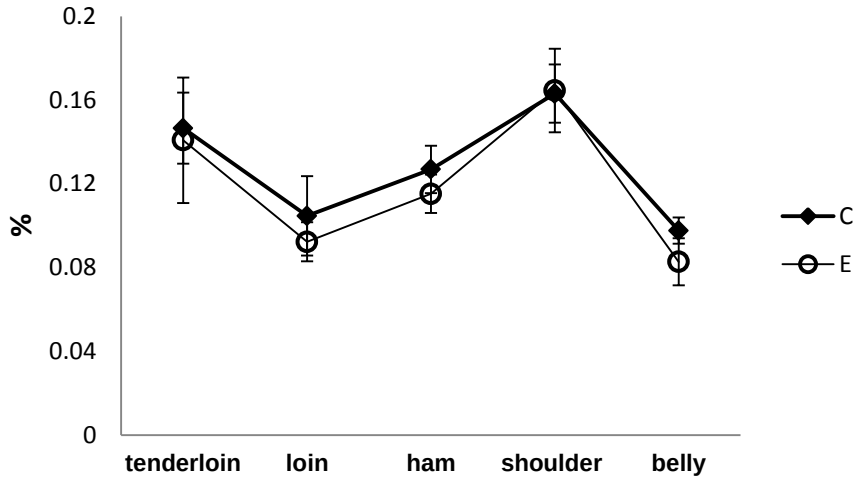
*Values with the different superscript in the same raw are statistically different (P <0.05)
C-Control, E- Control + chromium picolinate, CrPic- chromium picolinate

The five main cuts, namely ham, loin, shoulder, belly and tenderloin, were considered for proximate (crude protein, crude fat) and cholesterol analysis to determine the influence of CrPic on carcass quality (Figure 2). CP concentrations increased for all anatomical parts belonging to E group, but for tenderloin and ham, treatment E differed (P = 0,0342 for tenderloin and P = 0.0024 for ham) from treatment C. The opposite tendency was observed for crude fat concentrations in all analysed samples (decreased values for E group), but differences (P = 0.0357 for belly and P = 0.0305 for ham) were noticed for belly and ham samples. The values obtained for cholesterol level in Cr-supplemented group samples, were lower than samples from C group, but only numerically (P >0.05) (Figure 3).



C-Control, E- Control + chromium picolinate

Figure 2 Crude protein and crude fat concentrations in five anatomical parts of pigs fed two diets differing in chromium picolinate concentration



C-Control, E- Control + chromium picolinate

Figure 3 Cholesterol concentrations in five anatomical parts of pigs fed two diets differing in chromium picolinate concentration

Carcass quality was influenced by Cr supplementation. The positive effect of supplemented Cr in increasing the proportion of muscle to fat has been reported in several studies (Page *et al*., 1993; Wang & Xu, 2004). A plausible theory is that a low molecular weight Cr binding protein (chromodulin) is linked to the action of the insulin receptor, glucose entry is increased into adipocytes, lipogenesis is increased and the net fatty acid release decreases (McNamara & Valdez, 2005). The results of the current study indicate that Cr as CrPic had no effect on the cholesterol levels of the five anatomical parts. Research studies highlighted the positive effect of Cr in decreasing the blood level cholesterol (Vincent, 2000), but other researchers did not find any effect of Cr on lipid metabolism (Rabinowitz *et al*., 1983).

For fatty acids characterization, the authors considered the anatomical parts with fat content being significantly (P >0.05) lower in E group compared with C (Table 4). No significant differences were noticed between groups regarding the quality of fat.

Regarding meat fatty acids composition, the current results agree with findings of other researchers (Jackson *et al*., 2009), who considered that Cr supplementation did not influence the fatty acid characteristics, because the profile is a reflection of the dietary fatty acids. A systematic review of eighteen human studies of the effect of Cr supplementation on lipid profiles showed a statistically heterogeneity of meta-analyses. The conclusion of the study was that there was no beneficial effect of Cr supplementation on lipid metabolism (Balk *et al*., 2007). Choi *et al*. (1998) considered that Cr increased in vitro lipolytic activity in adipose tissue of pig, without effects on lipogenesis, while Xi *et a*l. (2001) showed that Cr supplements may decrease fat accretion through inhibiting lipogenesis.

Table 5 shows the amino acid composition in tenderloin and ham samples (the only two anatomical parts with significant increased protein concentration compared to C group). A lack of published data was noticed about the influence of Cr supplements on amino acid concentrations of pork. In the current study, the experimental group had higher levels (P <0.05) of dispensable, semi-essential and essential amino acids, including glutamic acid, arginine, valine (P <0.05) and serine, tyrosine (P <0.01), in tenderloin and ham. Lysine, cystine, methionine and arginine are the essential amino acids for pigs (Habeanu *et al*., 2011). In the tenderloin, significantly higher levels (P <0.05) of arginine and lysine, and only arginine for ham, were registered for the experimental group. The sum of essential amino acids increased (P <0.05) compared with C group, for both types of cuts. C and E diets had the same level of amino acids and no differences were reported regarding average daily intake. In this context, it can be considered that the positive effect registered on the amino acid profile of E group is the influence of CrPic supplement.

Amino acids are the basic units of proteins and determine the quality and flavour of the meat (Cai, 2010). Cai *et al*. (2010) and Conde-Aguilera *et al*. (2014) reported high contents of glutamic acid, aspartic acid, lysine, leucine and arginine in the *longissimus dorsi* muscle of pigs, which is similar to the current results. Evans & Bowman (1992) showed that Cr supplementation in rat diets led to an increased uptake of glucose and amino acids in the skeletal muscle. These findings were attributed to the change in insulin parameters which are Cr related (Amata, 2013). Lien *et al* (2001) suggest that Cr supplementation increases the activity of insulin, which stimulates amino acid transport and protein synthesis in muscle cells.

Table 4 Fatty acid composition of belly and ham samples

Fatty acid (% of total FAME*)		Belly				Ham			
		C	E	SEM	P-value	C	E	SEM	P-value
Lauric acid	(C12:0)	0.15	0.14	0.004	0.207	0.15[b]	0.22[a]	0.02	0.014
Myristic acid	(C14:0)	1.94	1.82	0.05	0.247	1.76	1.96	0.07	0.127
Pentadecanoic acid	(C15:0)	0.24	0.21	0.02	0.510	0.13	0.16	0.01	0.355
Pentadecenoic acid	(C15:1)	0.09	0.19	0.03	0.132	0.57	0.55	0.06	0.842
Palmitic acid	(C16:0)	27.40	25.98	0.43	0.195	26.71	26.73	0.32	0.951
Palmitoleic acid	(C16:1)	4.01	3.47	0.19	0.160	4.10	4.07	0.15	0.940
Heptadecanoic acid	(C17:0)	0.35	0.38	0.04	0.790	0.40	0.40	0.04	0.929
Heptadecenoic acid	(C17:1)	0.36	0.38	0.04	0.797	0.36	0.36	0.03	0.928
Stearic acid	(C18:0)	11.90	12.54	0.38	0.448	10.47	10.21	0.30	0.715
Oleic acid	(C18:1)	42.01	41.67	0.55	0.898	40.69	40.36	0.62	0.950
Linoleic acid	(C18:2)	10.11	11.37	0.41	0.130	10.62	11.77	0.42	0.181
Linolenic acid	(C18:3n-3)	0.34	0.37	0.01	0.240	0.31	0.34	0.02	>0.99
Eicosadienoic acid	(C20:2n-6)	0.53	0.53	0.02	0.963	0.51	0.44	0.02	0.155
Eicosatrienoic acid	(C20:3n-6)	0.34	0.35	0.01	0.843	0.37	0.36	0.02	0.844
Eicosatrienoic acid	(C20:3n-3)	0.04	0.02	0.01	0.821	0.20	0.18	0.02	0.298
Arachidonic acid	(C20:4n-6)	0.30	0.52	0.06	0.080	1.66	1.40	0.15	0.432
Docosatetraenoic acid	(C22:4n-6)	-	-	-	-	0.28	0.25	0.02	0.272
Other fatty acids		0.15	0.05	0.57	0.401	0.58	0.23	0.04	0.175
TOTAL									
Σ Saturated acids		41.72	41.06	0.57	0.602	39.61	39.72	0.53	0.931
Σ Unsaturated acids		58.14	58.88	0.47	0.550	59.69	60.08	0.53	0.739
Σ n-6		11.28	12.77	1.53	0.104	13.45	14.23	0.51	0.488
Σ n-3		0.38	0.39	0.46	0.770	0.51	0.52	0.01	0.662
Ratio n-6/n-3		30.06	32.77	0.57	0.418	26.44	27.23	0.67	0.591

*FAME: fatty acid ester methyl, C-Control, E- Control + chromium picolinate, [ab] Values with the different superscript in the same row are statistically different (P <0.05)

Conclusions

The results of the study indicate that Cr supplements (200 ppb) improve certain aspects of nutrient balance parameters and pork quality. Cr supplementation increased the rate of protein deposition (tenderloin and ham) and reduced the rate of fat deposition (belly and ham). The concentrations of essential amino acids in tenderloin and ham samples of Cr supplemented group were improved, indicating the potential of using Cr to develop functional foods. The mechanisms underlying these effects are not fully understood, and more research is necessary to evaluate the consistency of these results.

Table 5 Amino acid composition in tenderloin and ham samples

Amino acids (% DM)	Tenderloin				Ham			
	C	E	SEM	P-value	C	E	SEM	P-value
Aspartic acid	8.30	8.95	0.18	0.0640	9.10	9.40	0.125	0.2672
Glutamic acid	15.59^{b}	17.15^{a}	0.34	0.0059	23.88^{b}	19.47^{a}	0.900	0.0010
Glycine	4.28	4.63	0.11	0.1269	4.26	4.56	0.204	0.5058
Alanine	5.35^{b}	5.80^{a}	0.11	0.0437	5.72	6.06	0.097	0.0677
Serine	4.83^{b}	5.30^{a}	0.09	0.0475	2.63^{b}	3.48^{a}	0.164	0.0001
Arginine	7.47^{b}	8.09^{a}	0.16	0.0422	7.45^{b}	8.14^{a}	0.159	0.0112
Tyrosine	3.08	3.30	0.06	0.0778	3.25^{b}	3.97^{a}	0.144	0.0004
Cystine	1.15	1.14	0.02	0.8421	0.89	0.85	0.030	0.5005
Threonine	3.90	4.11	0.07	0.1764	4.51	4.79	0.082	0.0829
Valine	3.17	3.42	0.09	0.1718	6.06^{b}	6.92^{a}	0.210	0.0244
Phenylalanine	2.50	2.55	0.06	0.7585	3.80	3.97	0.056	0.1312
Isoleucine	4.04^{b}	4.60^{a}	0.13	0.0273	4.43	4.56	0.065	0.3233
Leucine	7.13^{b}	7.85^{a}	0.18	0.0381	8.05	8.43	0.119	0.1060
Lysine	6.77^{b}	7.71^{a}	0.23	0.0298	8.02	8.02	0.096	0.9724
Methionine	1.38	1.47	0.03	0.2050	1.58	1.63	0.079	0.7784
Other amino acids	21.06^{b}	13.95^{a}	1.74	0.027	6.38	5.76	0.460	0.5423
TOTAL								
Σ dispensable amino acids	33.52^{b}	36.53^{a}	0.71	0.0175	42.94^{b}	39.48^{a}	0.662	<0.0001
Σ Semi-essential amino acids	16.53^{b}	17.83^{a}	0.33	0.0391	14.22^{b}	16.44^{a}	0.445	0.0005
Σ Essential amino acids	28.89^{b}	31.69^{a}	0.71	0.0351	36.45^{b}	38.32^{a}	0.251	0.0404

C-Control, E- Control + chromium picolinate, ^{ab}Values with the different superscript in the same row are statistically different (P <0.05)

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Authors' Contributions

AEU and IV performed the design of study, interpretation of data and drafted the manuscript. TDP, MH, GMC, MR, MO, IV participated at acquisition of data. MH revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

Conflict of Interest Declaration

The authors of the manuscript have no conflict of interest to declare

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