

Suhuai suckling piglet ionomic-metabolome responses to different dietary copper level in antibiotic free creep feed

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Research

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Abstract

Background: High levels of dietary copper may enhance co-selection in favor of antibiotic resistant bacteria and promote the spread of antibiotic resistance. This possibility necessitates further assessment of the safety and utility of supplementing feed with copper.

Methods: Eighteen Suhuai sows at second parity were divided into 3 experimental groups in which their 180 suckling piglets had access to antibiotic free creep feed with different levels of copper supplementation: low copper diet (LC, 6 mg·kg⁻¹), control diet (CON, 20 mg·kg⁻¹), and high copper diet (HC, 300 mg·kg⁻¹), which was offered ad libitum from day 14 until weaning at day 40. The growth performance, serum biochemical parameters, ionic profiles (hair, serum, and feces), fecal significant metabolites of suckling piglets, and the correlations were analyzed.

Results: In HC group, the average daily gain (ADG) and average daily feed intake (ADFI) increased during d 14 to 28 ($P < 0.05$), but ADG was decreased with extension of feeding time (d 29 to 40) ($P < 0.01$). Compared with the CON group, the tumor necrosis factor- α (TNF- α) ($P < 0.05$) was decreased while total antioxidant capacity (T-AOC) ($P < 0.05$) was increased in HC group. The hair Na ($P < 0.01$) and K ($P < 0.01$) concentrations were increased in HC group than CON group; hair Cu ($P < 0.01$), fecal Cu ($P < 0.01$) increased in the HC group than LC group. The hair Na and K were negatively correlated with serum TNF- α and fecal inosine ($P < 0.05$), while positively correlated with serum insulin-like growth factors-1 (IGF-1) and T-AOC ($P < 0.05$); the hair Cu was negatively correlated with serum malondialdehyde (MDA), total bile acid (TBA) and fecal putrescine, glucose-6-phosphate, fumaric acid ($P < 0.05$); the fecal Cu was positively correlated with serum growth hormone ($P < 0.05$), negatively correlated with fecal methionine, pantothenic acid, and uracil ($P < 0.05$). Further metabolic pathway enrichment analysis showed that the hair Cu was negatively correlated with phenylalanine and tyrosine metabolism, and mitochondrial electron transport chain pathways; fecal Cu was negatively correlated with betaine metabolism, and pantothenate and CoA biosynthesis pathways.

Conclusions: Dietary 300 mg·kg⁻¹ copper altered the ion balance and further affected the body's redox balance state and metabolic homeostasis, which adverse to the health of piglets; dietary 20 mg·kg⁻¹ copper maintain ion homeostasis and suitable to meet the nutritional needs of suckling piglets than 6 mg·kg⁻¹ dietary copper.

Background

To prevent the selection for and the spread of antibiotic resistance, Sweden in 1986, then Denmark, the United Kingdom and many countries of the European Union banned the use of antibiotic agents as growth promoters in animal husbandry [1, 2]. As a consequence, many studies have concentrated on dietary alternatives to antibiotics, with considerable interest in supplementing diets with high levels of copper; however, using high levels of copper as an alternate to antibiotic growth promoters in animal production to control the emergence and propagation of antibiotic resistance has yielded limited success.

Antibiotic resistance has not yet been eliminated in these European Union countries despite the introduced bans. Previous studies have shown that heavy metals used in animal farming may contribute to the spread of antibiotic resistance through co-selection [3], and Cu-induced spread of resistance to antibiotics relevant to veterinary and human medicine was discovered in 2002 [4], while many studies have proved that high levels of dietary copper may further enhance co-selection of antibiotic resistant bacteria [1, 5, 6]. Restrictions on trace mineral additives to livestock diets have already been implemented in Europe, but in China's swine industry, copper supplements are generally introduced at higher concentrations than required by the US National Research Council (NRC), which has suggested dietary Cu requirements for 5 to 25 kg nursery pigs and growing pigs of approximately 3 to 6 mg·kg⁻¹ [7]; these copper levels may be met by Cu in pig feed ingredients [8], or at higher levels (up to 16.5 mg·kg⁻¹) to compensate for dietary factors that could reduce absorption [9]. Dietary high level copper and their released ions, Cu²⁺, are believed to be one of the most important pollution-causing metals [10], while in China, use of antibiotics in swine production has only recently been banned [11], suggesting that it is time to be concerned about excess supplementation of feed with Cu in feed as well.

Copper is an essential trace element for pigs, and harmful to their health when dietary copper is deficient [12], and pharmacological Cu is routinely included to pig diets due to its pronounced promotion of growth [13–17]. Our previous studies have suggested that high levels of dietary copper (240 and 300 mg·kg⁻¹) affect the health of rats and suckling piglets by altering the composition of their intestinal microflora [18, 19], and we identified the need to properly evaluate whether excessive feed supplementation with Cu influences the absorption and utilization of other elements and metabolites, thus further impacting growth and health. Research on the growth-promotion effect of dietary copper has been mostly conducted in China and the United States, which did not ban the use of antibiotics from 2006 to 2014 [20]. In these studies of high levels of both copper and antibiotics in feed, it has been hard to distinguish efficiency effects came from copper supplements, because various antibiotics or antibacterial agents in combination with CuSO₄ have demonstrated an additive performance effect in pigs [21].

Suhuai suckling piglets were used as our experiment model. Hybridization of Huai sows and Yorkshires began in 1958, and through decades of breeding Suhuai pigs were approved as a new breed in 2011 by China's national commission of animal genetic resources [19]. Copper supplementation in Suhuai piglets usually follows commercial standards despite the lack of breed-specific studies.

This study assumes a new perspective to evaluate the use of high levels of dietary copper, with the aim of investigating their effects on the ion balance and health status of Suhuai suckling piglets, to assess the safety and utility of high levels of copper in antibiotic free creep feed.

Materials And Methods

Animal, Housing, Diets, and Sampling

A total of 180 piglets (average initial weight approximately 1.11 ± 0.18 kg) from 18 multiparous Suhuai sows (second pregnancy) were assigned to 2 rooms (9 litters/room, 10 piglets per litter). In each litter, all piglets were selected based on similar body weight (BW), BW and sex were balanced among the piglets, and all piglets were individually weighed 72 h after farrowing. All 18 litters were randomly divided into three groups (6 litters/treatment): (i) low copper diet (LC, $6 \text{ mg}\cdot\text{kg}^{-1}$) containing no supplemental Cu; (ii) control diet (CON, $20 \text{ mg}\cdot\text{kg}^{-1}$); or (iii) high copper diet (HC, $300 \text{ mg}\cdot\text{kg}^{-1}$), with dietary copper supplementation by means of copper sulfate (CuSO_4). The piglets were trained to feed when 7 days old with a prefeeding period of 7 to 14 days, and the animal trials were conducted over 26 days (14–40 days), and the corn/soybean based diets were supplied throughout the experiment, meeting the nutritional requirements of the NRC [7] (see Additional file 1, Table S1). The piglets were housed together with their sow during the experimental period and with free access to creep feed and water, all piglets hindered to have access and eat the sow feed [19].

Each litter was monitored three times a day, and creep feeders were refilled as needed. Wet creep feed was removed, dried, and weighed and feeders were refilled approximately every 8 h. Creep feed consumption were recorded daily. Individual pigs were weighed at d 14, 28, and 40 days post farrowing. Piglet BW and creep feed consumption were used to calculate ADG and ADFI. The diarrhea rate of piglets was recorded daily and calculated as follows: Diarrhea rate (%) = the number of pigs with diarrhea \times diarrhea days / (the total number of pigs \times experiment days) \times 100%, which the “number of pigs with diarrhea” was defined as the number of piglets with diarrhea was observed each day [22].

Before weaning, three litters were chosen within each treatment. Hair, feces and blood samples were harvested from four piglets/litter, selected based on average body weight (half male and female). Blood samples were stored in glass tubes with no anticoagulant and were allowed to clot at 4°C before harvest of serum by centrifugation (15 min at 3500 rpm). Serum, hair and fecal samples were stored at -80°C for subsequent analyses [18].

Serum Biochemical Parameters Analysis

Serum concentrations from piglets were detected for growth hormone (GH), IGF-1, leptin, and TNF- α using the enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All examinations were performed according to the manufacturer’s instructions [19].

Element Analysis

Thirteen elements including macro (Ca, Mg, Na, K, P), micro (Fe, Cu, Mn, Zn, Cr) and toxic (Pb, Al, Ni) elements were measured in hair, serum, and feces using inductively coupled plasma optical emission spectrometry (ICP-OES) (PerkinElmer, USA). Hair (200 μg), serum (1 mL), and dried feces (500 μg) were placed in a tube with 10 mL of a mixture of nitric acid (Guaranteed reagent, GR) and perchloric acid (GR) (3:1 v/v). After digestion overnight, tubes were heated from 100°C to 240°C over approximately 3 h, and

the resulting digests brought to constant volume with double distilled deionized water [18, 23]. The standard liquid of Ca, Mg, Na, K, and P ($1000 \mu\text{g}\cdot\text{mL}^{-1}$) were mixed to prepare a 5 mL mixed standards with $0.5 \text{ mol}\cdot\text{L}^{-1} \text{HNO}_3$. Take 0.5 mL mixed standard of Fe, Cu, Mn, Zn and Cr ($1000 \mu\text{g}\cdot\text{mL}^{-1}$), constant volume to 5 mL secondary mother liquor ($100 \mu\text{g}\cdot\text{mL}^{-1}$), then using secondary mother liquor prepare 10 mL standard with $0.5 \text{ mol}\cdot\text{L}^{-1} \text{HNO}_3$. Take 0.5 mL mixed standard of Pb, Al, and Ni ($1000 \mu\text{g}\cdot\text{mL}^{-1}$), constant volume 2 times and prepare 5 mL secondary mother liquor ($10 \mu\text{g}\cdot\text{mL}^{-1}$), then prepare 10 mL standard with $0.5 \text{ mol}\cdot\text{L}^{-1} \text{HNO}_3$. According to the instrument software settings, the determination of each standard of solution's absorbance value, each sample determination of repeated three times, get the element of standard curve, according to the standard curve of the conversion of each element content in the samples.

Fecal metabolite profiles analysis

The fecal samples preparation for gas chromatography-mass spectrometer analysis and data acquisition and processing were consistent with what we've already published (see Additional file 1) [19]. The fecal metabolites with variable important projection (VIP) value > 1.0 and one-way analysis of variance (ANOVA) P values < 0.05 were considered as significant metabolites among the three dietary groups [19].

Statistical analysis

Growth performance and serum biochemical parameters as well as hair ionic profiles were compared among the groups by means of a one-way ANOVA after any needed normal test processing and data conversion. For data found not to possess a normal distribution, the nonparametric Kruskal-Wallis test was utilized. Correlation analysis was conducted using a Karl-Pearson correlation test. Significant differences were declared when $P < 0.05$. The statistical analysis was done using SPSS Statistics Version 22 (<https://www.ibm.com/analytics/spss-statistics-software>) [19].

Results

Growth Performance

During phase 1 (d 14 to 28) the ADG ($P < 0.05$) and ADFI ($P < 0.05$) in the HC group increased, compared with the LC or CON groups (Table 1), and with extension of feeding time ADG ($P < 0.01$) was decreased in the HC group compared with the LC and CON groups (d 29 to 40). The diarrhea rate of the HC group was significantly decreased ($P < 0.05$) compared with LC groups for the entire experimental period.

Table 1
Effects of dietary copper on the growth performance of suckling piglets (Mean \pm SD)

Items	Cu supplementation, mg·kg ⁻¹ diet			SEM	P-value
	LC (6)	CON (20)	HC (300)		
Phase 1, d 14 to 28					
ADG, g	170.77 \pm 30.10 ^b	183.28 \pm 34.81 ^{ab}	197.45 \pm 18.92 ^a	6.89	< 0.05
ADFI, g	49.77 \pm 4.23 ^{ab}	45.50 \pm 5.50 ^b	62.44 \pm 15.23 ^a	2.76	< 0.05
G:F	3.47 \pm 0.76	4.12 \pm 1.11	3.29 \pm 0.77	0.22	0.26
Phase 2, d 29 to 40					
ADG, g	202.95 \pm 26.11 ^a	214.92 \pm 16.30 ^a	163.68 \pm 36.74 ^b	8.11	< 0.05
ADFI, g	120.06 \pm 16.06	117.88 \pm 18.14	118.21 \pm 21.46	4.14	0.98
G:F	1.73 \pm 0.41	1.86 \pm 0.29	1.45 \pm 0.51	0.10	0.26
Overall, d 14 to 40					
ADG, g	185.92 \pm 25.98	198.02 \pm 20.53	183.36 \pm 13.28	2.76	0.44
ADFI, g	74.28 \pm 4.61	72.92 \pm 9.03	81.30 \pm 13.45	2.33	0.31
G:F	2.51 \pm 0.39	2.76 \pm 0.53	2.31 \pm 0.45	0.11	0.27
Diarrhea rate, %	8.74 \pm 3.99 ^a	6.55 \pm 5.37 ^{ab}	2.22 \pm 1.18 ^b	1.09	< 0.05
^{a,b} Values within a row without a common superscript letter are significantly different ($P < 0.05$)					

Serum Biochemical Parameters

Compared with other groups, serum growth hormone of the HC group ($P < 0.05$) increased, while albumin ($P < 0.05$) was significantly decreased (Table 2). Compared with the CON group, ALT ($P < 0.05$) and AST ($P < 0.01$) as well as MDA ($P = 0.05$) for the LC group increased, and T-AOC ($P < 0.05$) also increased in the HC group, while TNF- α ($P < 0.05$) decreased in HC group. Compared with the CON group, serum BUN ($P < 0.01$) and SOD ($P = 0.09$) increased in the LC and HC groups.

Table 2
Effects of dietary copper on serum biochemical parameters in suckling piglets (Mean \pm SD)

Items	Cu supplementation, mg·kg ⁻¹ diet			SEM	P-value
	LC (6)	CON (20)	HC (300)		
Growth related hormones					
Growth hormone, ng·mL ⁻¹	1.21 \pm 0.31 ^b	1.19 \pm 0.43 ^b	1.67 \pm 0.41 ^a	0.08	<0.05
IGF-1, U·mL ⁻¹	9.52 \pm 1.93	9.68 \pm 2.23	10.95 \pm 2.07	0.38	0.26
Leptin, ng·mL ⁻¹	5.87 \pm 0.71	5.79 \pm 1.11	5.26 \pm 1.59	0.21	0.46
Inflammatory cytokine					
TNF- α , ng·L ⁻¹	70.43 \pm 13.96 ^{ab}	80.03 \pm 24.82 ^a	58.37 \pm 17.78 ^b	3.69	<0.05
Oxidative, antioxidant enzymes					
T-AOC, U·mL ⁻¹	2.14 \pm 0.65 ^{ab}	1.64 \pm 0.43 ^b	2.33 \pm 0.76 ^a	0.12	<0.05
MDA, nmol·mL ⁻¹	3.46 \pm 0.77 ^a	2.83 \pm 0.53 ^b	3.01 \pm 0.46 ^{ab}	0.11	0.05
SOD, U·mL ⁻¹	142.93 \pm 14.00	149.94 \pm 7.47	138.51 \pm 13.63	2.20	0.09
Hepatic function					
ALT, U·L ⁻¹	11.47 \pm 4.38 ^a	7.59 \pm 2.00 ^b	8.55 \pm 3.17 ^{ab}	0.63	<0.05
AST, U·L ⁻¹	9.17 \pm 2.28 ^a	6.02 \pm 1.73 ^b	7.06 \pm 2.08 ^{ab}	0.41	<0.01
TBA, μ mol·gprot ⁻¹	91.30 \pm 43.07 ^a	53.74 \pm 24.94 ^b	55.82 \pm 18.57 ^b	6.14	<0.05
T-CHOL, nmol·L ⁻¹	3.27 \pm 0.59	3.68 \pm 0.95	3.06 \pm 0.60	0.14	0.15
Albumin, g·L ⁻¹	27.23 \pm 4.18 ^a	27.01 \pm 3.41 ^a	23.83 \pm 2.16 ^b	0.62	<0.05
Renal function					
BUN, mmol·L ⁻¹	3.98 \pm 0.60 ^b	4.62 \pm 0.62 ^a	3.68 \pm 0.84 ^b	0.14	<0.01
Creatinine, μ mol·L ⁻¹	59.22 \pm 12.98	70.36 \pm 14.57	60.39 \pm 15.20	2.58	0.14
BUN/Creatinine	70.40 \pm 19.79	67.82 \pm 14.77	65.85 \pm 25.43	3.44	0.88
^{a,b} Values within a row without a common superscript letter are significantly different (P < 0.05).					

Ionic profiles

In hair, Mg ($P < 0.05$) and Al ($P = 0.07$) in the CON group increased compared with the LC and HC groups. Na ($P < 0.01$) concentration increased in the HC group compared with other groups. Compared with the CON group, K ($P < 0.01$) in the HC group was increased, while P ($P < 0.05$), Mn ($P < 0.05$) and Fe ($P = 0.07$) decreased. Cu ($P < 0.01$) concentration in the LC group decreased compared with the HC group (Figure 1a, b and c). In serum, Ca ($P = 0.05$) and P ($P < 0.05$) concentrations in the HC group increased compared with the LC group, and Mg ($P < 0.05$) increased in CON group (Figure 1d). In feces, Cu ($P < 0.01$) concentration increased in the HC group compared with the LC group (Figure 1h).

Fecal significant metabolites profiles

Our previous analysis showed that a total of 47 significant fecal metabolites were identified among the three dietary group (see Additional file 1, Table S2) [19]. The effects of dietary copper level on normalized relative abundance of fecal significant metabolites were shown in Figure 2. Compared with HC group, the relative abundance of leucine, proline, tyrosine, phenylalanine, methionine (Figure 2a), fructose-6-phosphate, mannose-6-phosphate, glucose-6-phosphate (Figure 2b), and 2-hydroxyglutaric acid (Figure 2c) were increased in CON group ($P < 0.05$). The relative abundance of remaining 23 significant metabolites in CON group were increased than other groups ($P < 0.05$).

Changes in correlation patterns among elements in hair, serum and feces

Correlation patterns among elements at different dietary copper levels are presented in Figure 3. In hair, the number of correlations in the macro-micro category (upper-left, Figure 3a, b and c) in the CON group was greater than for other groups. Compared with the CON group, the correlations of Cu = f(Mg), Mn = f(Na, K), and Zn = f(K) were negligible in both the LC and HC groups. The correlations of Fe = f(Mg), Cu = f(Na, K), Mn = f(Na, K), Zn = f(Na, K), and Zn = f(Ca, P) were negligible in the HC group. In the toxic-micro and toxic-macro categories (upper-right and bottom left, Figure 3a, b and c), Ni = f(Cr, Cu) and Al = f(Na, K) were negligible in both the LC and HC groups compared with the CON group, while Al = f(Cr) and Pb = f(-Cu, -Na) appeared in the HC group. In serum, in the macro-micro category (upper-left, Figure 3d, e and f), Fe = f(Ca, Mg, Na, P), Cu = f(Ca), Mn = f(Ca, Mg) and Cr = f(P) were negligible, while Cu = f(Na, P) appeared in both the LC and HC groups. No significant positive correlations were observed between toxic and micro or macro elements in the CON group. In feces, the negative correlations of Ca and P with most micro and toxic elements were negligible, while the positive correlation of Mg and K were appeared when dietary copper levels increased from 20 to 300 mg·kg⁻¹.

Correlation between ionic profiles and serum biochemical parameters

The correlation between ionic profiles and serum biochemical parameters were presented in Figure 4. Growth hormone was positively correlated with fecal Cu and Zn ($P < 0.05$); serum TNF- α was negatively correlated with hair Na and K ($P < 0.05$) and positively correlated with fecal Cr ($P < 0.05$); serum MDA was negatively correlated with hair Fe, Cu, and Mn ($P < 0.05$) and positively correlated with fecal Fe, Cr and Pb ($P < 0.05$); serum T-AOC was positively correlated with hair Na and K ($P < 0.05$) and negatively correlated with fecal Mg, P, and Zn ($P < 0.05$); serum TBA was negatively correlated with hair and serum Cu and

fecal Na ($P < 0.05$) and positively correlated with hair Pb ($P < 0.05$); serum albumin was positively correlated with fecal Fe ($P < 0.05$); serum BUN was negatively correlated with fecal Cu ($P < 0.05$).

Correlation between ionic profiles and fecal significant metabolites

The correlation between ionic profiles and fecal significant metabolites were presented in Figure 5. Hair Na and K were negatively correlated with inosine ($P < 0.05$); hair Cu was negatively correlated with putrescine, 2-aminobutyric acid, glucose-6-phosphate, mannose-6-phosphate, inosine, 2-methylbutanedioic acid, fumaric acid, and oxalic acid ($P < 0.05$); serum Ca, Mg, and P were negatively correlated with arginine, homoserine, ornithine, fructose-6-phosphate, 9-(Z)-Octadecenoic acid, 9,12-(Z,Z)-Octadecadienoic acid, 2-hydroxyglutaric acid, and pantothenic acid ($P < 0.05$); fecal Cu was negatively correlated with methionine, malic acid, pantothenic acid, and uracil ($P < 0.05$).

The significant fecal metabolites correlated with ionic profiles were used for further metabolic pathway enrichment analysis (Figure 6). The hair Cu was negatively correlated with nucleotide sugars metabolism, starch and sucrose metabolism, aspartate metabolism, phenylalanine and tyrosine metabolism, and mitochondrial electron transport chain pathways; serum Ca, Mg, and P were negatively correlated with Urea cycle, arginine and proline metabolism, and α -linolenic acid and linoleic acid metabolism pathways; fecal Cu was negatively correlated with β -alanine metabolism, betaine metabolism, malate-aspartate shuttle, and pantothenate and CoA biosynthesis pathways.

Discussion

Copper plays an important role in pigs for the synthesis of hemoglobin and activation of several oxidative enzymes necessary for normal metabolism [15]. Previous studies have shown that higher nutritional levels of Cu (as CuSO_4) at concentrations of 100 to 250 $\text{mg}\cdot\text{kg}^{-1}$ improved growth performance in young pigs [24–26]. The National Research Council (NRC) suggests that the dietary Cu requirement for 5 to 25 kg nursery pigs and growing pigs is approximately 3 to 6 $\text{mg}\cdot\text{kg}^{-1}$ [7], suggesting that Cu requirements may be met by Cu present in feed ingredients when piglets are fed a corn-soybean base diet [8]. In our study, LC (6 $\text{mg}\cdot\text{kg}^{-1}$ Cu) seems unable to meet the nutritional needs of suckling piglets when antibiotics are withdrawn from creep feed because the G:F value decreased compared with the CON group (20 $\text{mg}\cdot\text{kg}^{-1}$ Cu), and the diarrhea rate significantly increased compared with the HC group (300 $\text{mg}\cdot\text{kg}^{-1}$ Cu) group. We also observed that 300 $\text{mg}\cdot\text{kg}^{-1}$ of dietary Cu enhanced ADG and ADFI during days 14 to 28, but that ADG and the G:F value decreased during days 29 to 40. Taken together with the lack of a significant growth response to high levels of Cu over the entire experimental period, these results suggest that high levels of dietary copper can promote short term growth. It has been believed that the growth-promoting effects of copper are related to the GH axis and might be generated by the stimulation of GH secretions [27]. Our study observed that serum GH increased in the HC group, similar to the results of a previous study which showed that high copper levels (100 to 300 $\text{mg}\cdot\text{kg}^{-1}$) can increase serum GH concentrations in weaning pigs [28]. However, high levels of copper did not affect the serum IGF-1 in our

study, unlike Wang's previous study, which found that dietary copper ($250 \text{ mg}\cdot\text{kg}^{-1}$, CuSO_4) increased serum IGF-1 concentrations on days 20 and 40 for weanling pigs, with the rise in serum IGF-1 partly due to increased feed intake [29]. In our study ADFI was no longer significantly increased in the HC group during days 29 to 40. When piglets were fed a $20 \text{ mg}\cdot\text{kg}^{-1}$ Cu diet, they tended to gain more and eat less among the three groups across the entire experimental period, while their diarrhea rate was within an acceptable range. $20 \text{ mg}\cdot\text{kg}^{-1}$ of Cu in an antibiotic free diet thus would be adequate to meet requirements of suckling piglets.

To investigate the effects of high levels of dietary copper on the health of piglets, serum biochemical parameters were further analyzed. In the HC group, TNF- α concentrations decreased. This result suggested that high levels of copper may have a potential anti-inflammatory effect when antibiotics are withdrawn from the diet. In the current study, serum T-AOC concentration increased in the HC group, reflecting the status of antioxidants in the serum [30], suggesting that high levels of copper in the diet had an obvious effect on the antioxidative status of suckling piglets. In our study, compared with other groups, MDA decreased and SOD tended to increase in the CON group, suggesting that dietary concentrations of $20 \text{ mg}\cdot\text{kg}^{-1}$ Cu enhance the ability to resist oxidative stress in piglets. However, SOD tended to decrease in the HC group, because high levels of copper may weaken the activation of SOD, which was in agreement with our previous study that found the mRNA levels of *Ccs* and *Sod1* genes were reduced by high levels of dietary copper ($240 \text{ mg}\cdot\text{kg}^{-1}$) in the liver of SD rats (see Additional file 1, Figure S1). Our results also suggested that liver dysfunction might occurred in piglets in the LC and HC groups because of observed elevations in ALT, AST and bile acid levels in serum, considered valuable biomarkers for the diagnosis of hepatic disease [31–33]. Kidney function seems to be maintained in all piglets as evidenced by the fact that BUN/creatinine levels were not affected by the level dietary copper, which is considered a marker for identifying acute kidney injury (AKI) [34].

Viewing human hair as an excretory system for trace metals, and considering that concentrations of these elements in hair are reported to be correlated with the diagnosis of various diseases [35], hair mineral analysis has become an interesting diagnostic tool in assessment of health and nutritional status [36, 37]. In our study, of the 13 elements tested, concentrations of eight elements in hair were affected by levels of dietary copper, with hair Na, K, and Cu increasing in the HC group, suggesting that Na-K balance changes and reabsorption decreased. Concentrations in hair Fe, Mn and Al increased in the CON group more than in the HC group, and strong positive correlations between Fe-Al ($r= 0.851$) and Mn-Al ($r= 0.808$) were observed (see Additional file 1, Table S3). These elements share the same uptake mechanism (transferrin), and Fe, Mn and Al are related to inflammation in humans and animals [38], corroborated by an observed increase in TNF- α in the CON group. Noteworthy changes were seen in the correlation pattern between macro and micro or toxic elements with increased copper levels in the diet (Fig. 2). The number of positive correlations between macro-micro elements in hair and serum increased in the CON group compared with other groups (Fig. 2b and e); further, the absorption, utilization and excretion of many trace elements in animals are greatly affected by other trace elements [39], so these results suggest that dietary $20 \text{ mg}\cdot\text{kg}^{-1}$ Cu can maintain homeostasis in piglets due to maintenance of

the interactions between macro and micro elements [12]. Correlations between hair macro (Na, K) and micro (Cu, Mn, Zn) elements appear in the HC group (Fig. 2c), and as is well known, trace elements such as Cu, Zn and Mn are essential for normal growth, disease resistance, production and reproduction in farm animals [12], suggesting that a dietary dose of $300 \text{ mg}\cdot\text{kg}^{-1}$ Cu changed the balance of Na-K and affected the absorption and utilization of Cu, Mn, and Zn. We also observed that a negative correlation of toxic (Pb and Al) elements appeared in the HC group. These changes could reflect imbalance or adverse status in these elements [38], and marginal or severe element imbalances can be considered risk factors for several diseases [40]. These results suggest that a dietary dose of $300 \text{ mg}\cdot\text{kg}^{-1}$ Cu might have adverse effects on the health of suckling piglets.

To understand the relationship between the ion balance and health status of suckling piglets, correlations between ionic profiles and serum biochemical parameters were analyzed. Hair Na and K were positively correlated with IGF-1 and T-AOC, and negatively correlated with TNF- α , these results suggested that changes in Na-K balance induced by $300 \text{ mg}\cdot\text{kg}^{-1}$ of dietary Cu was related to the inflammatory response and antioxidant capacity. The concentrations of Fe in hair, serum, and feces were positively correlated with MDA, ALT, and albumin, a relationship between Fe concentrations and hepatic function has been reported [41], and $300 \text{ mg}\cdot\text{kg}^{-1}$ of dietary Cu may affect the absorption of Fe and further damage the hepatic function, inducing oxidative stress in suckling piglets. Concentrations of Cu in hair, serum, and feces were negatively correlated with MDA, TBA, and BUN, suggesting that changes in Cu induced by $300 \text{ mg}\cdot\text{kg}^{-1}$ of dietary Cu affects the hepatic function and causes oxidative damage [42]. Dietary Cu at a concentration of $300 \text{ mg}\cdot\text{kg}^{-1}$ increased growth hormone in suckling piglets was demonstrated by the correlation between fecal Cu and growth hormone. The concentrations of Zn in hair and feces were positively correlated with MDA, T-AOC, and creatinine, since Zn has the function of stabilizing cell membrane structure and protecting free radicals from oxidative damage [43, 44], and participates in the regulation of liver function and urea production. In general, dietary high level copper affected the ionic profiles which in turn adversely affects the health of suckling piglets.

As we know, fecal metabolites reflect the final status of animal digestion, absorption, and metabolism of feed nutrients [19]. Our previous analysis of fecal significant metabolites showed that the capacity of dietary monosaccharide and protein absorption decreased, and the level of organic acids were increased in suckling piglets those fed with $6 \text{ mg}\cdot\text{kg}^{-1}$ Cu diet (Fig. 2), these suggest that $6 \text{ mg}\cdot\text{kg}^{-1}$ Cu supplementation has an adverse effect on the health of piglets when antibiotic withdrawn from the feed [19]. In this study, we are concerned about changes in the composition of elements in hair, which reflect the body's metabolism changes [40], the correlation and enrichment analysis showed that the Na and K in hair which significantly affected by dietary copper were negatively correlated with fecal inosine (Fig. 5), consistent with the result that hair Na and K were negatively correlated with serum TNF- α (Fig. 4), due to the inosine is a purine metabolite and has a systemic anti-inflammatory effect [45]. The hair Cu was significantly increased in HC group than LC group, and negatively correlated with phenylalanine and tyrosine metabolism pathways (Fig. 6), dopamine β -hydroxylase (DBH) and phenylalanine hydrolase are two key enzymes in these pathways which its cofactor is copper [46, 47]. The hair Cu was negatively

correlated with mitochondrial electron transport chain pathway (Fig. 6), various enzymes in this pathway use copper as a cofactor, such as cytochrome c oxidase and nicotinamide adenine dinucleotide (NADH) dehydrogenase [46–48]. The activity of the electron transport chain is related to the generation of reactive oxygen species (ROS) and the body's redox state [49, 50], in this study, dietary $300 \text{ mg}\cdot\text{kg}^{-1}$ Cu enhanced mitochondrial electron transport chain pathway which promoted the formation of ROS and affecting the redox status of piglets, these verified by the results of negative correlation between hair Cu and serum MDA (Fig. 4).

The fecal Cu content mainly comes from the accumulation of unabsorbed copper in the diet. In our study, fecal Cu was negatively correlated with betaine metabolism pathway, betaine is an important methyl donor in the process of homocysteine synthesis of methionine and an important intermediate in the process of energy metabolism [51, 52], the process by which betaine provides methyl groups is mainly catalyzed by betaine homocysteine transferase (BHMT), a cytoplasmic enzyme that relies on zinc activation [53], due to the antagonism between copper and zinc [12], suggested that dietary high level copper hinder the absorption of zinc to a certain extent, and inhibit the activity of BHMT as well as the function of betaine methyl donor. In the process of betaine producing methionine, homocysteine is also a substrate for the enzyme action of BHMT, when the activity of BHMT decreases, the level of homocysteine in the blood rises, which has a certain relationship with vascular disease, thrombosis and renal dysfunction [54, 55], these suggested that $300 \text{ mg}\cdot\text{kg}^{-1}$ Cu inhibited the methyl supply capacity of betaine and further affected protein biosynthesis and the health of suckling piglets. Our previous study found that the level of pantothenic acid was decreased in HC group [19], the enrichment analysis in this study showed that the fecal Cu was negatively correlated with pantothenate and coenzyme A (CoA) biosynthesis pathway, pantothenic acid plays an important role in the process of decomposing carbohydrates, fatty acids and amino acids to produce energy [56]. The results of this experiment showed that dietary high level copper affected the digestion and absorption of diets by piglets, which in turn affected the biosynthesis of pantothenic acid and CoA, further affected carbohydrate, fat, amino acid, and energy metabolism of suckling piglets, leading to changes in metabolic homeostasis.

Conclusions

In conclusion, $300 \text{ mg}\cdot\text{kg}^{-1}$ of dietary copper promoted growth in the short term but exhibited a lack of a significant growth over the entire experimental period, and altered the ion balance of Na, K and Fe, Cu, Zn, which may damage the body's redox balance state and hepatic function of piglets, further affected the metabolism of carbohydrate, fat, and amino acid. $6 \text{ mg}\cdot\text{kg}^{-1}$ of dietary Cu seems unable to meet the nutritional needs of suckling piglets. $20 \text{ mg}\cdot\text{kg}^{-1}$ of dietary Cu can effectively improve antioxidant capacity, protect tissues from oxidative damage, and maintain homeostasis due to preservation of the interactions between macro and micro elements, and it seems suitable for meeting the needs and maintaining the health of suckling piglets. These analyses and results may help us understand the effects of micronutrient intake on animal and human health.

List Of Abbreviations

ADFI, average daily feed intake; ADG, average daily gain; ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; CoA, coenzyme A; DBH, dopamine β -hydroxylase; GH, growth hormone; GR, Guaranteed reagent. IGF-1, insulin-like growth factors-1; MDA, malondialdehyde; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; SEM, standard error of mean; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TBA, total bile acid; T-CHOL, total cholesterol; TNF- α , tumor necrosis factor- α .

Declarations

Ethics approval

This animal experimental protocol was implemented under the supervision of the Chinese Guidelines for Animal Welfare and Experimental Protocol and were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China (NJAU-CAST-2015-098).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Wen Yao, Feng Zhang and Weijiang Zheng designed and supervised the experiments. Feng Zhang and Yongqiang Xue conducted the experiments. Feng Zhang and Yongqiang Xue performed the data measurements and statistical data analysis. Wen Yao and Feng Zhang wrote and revised the manuscript. All authors have read and approved the final manuscript.

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Figures

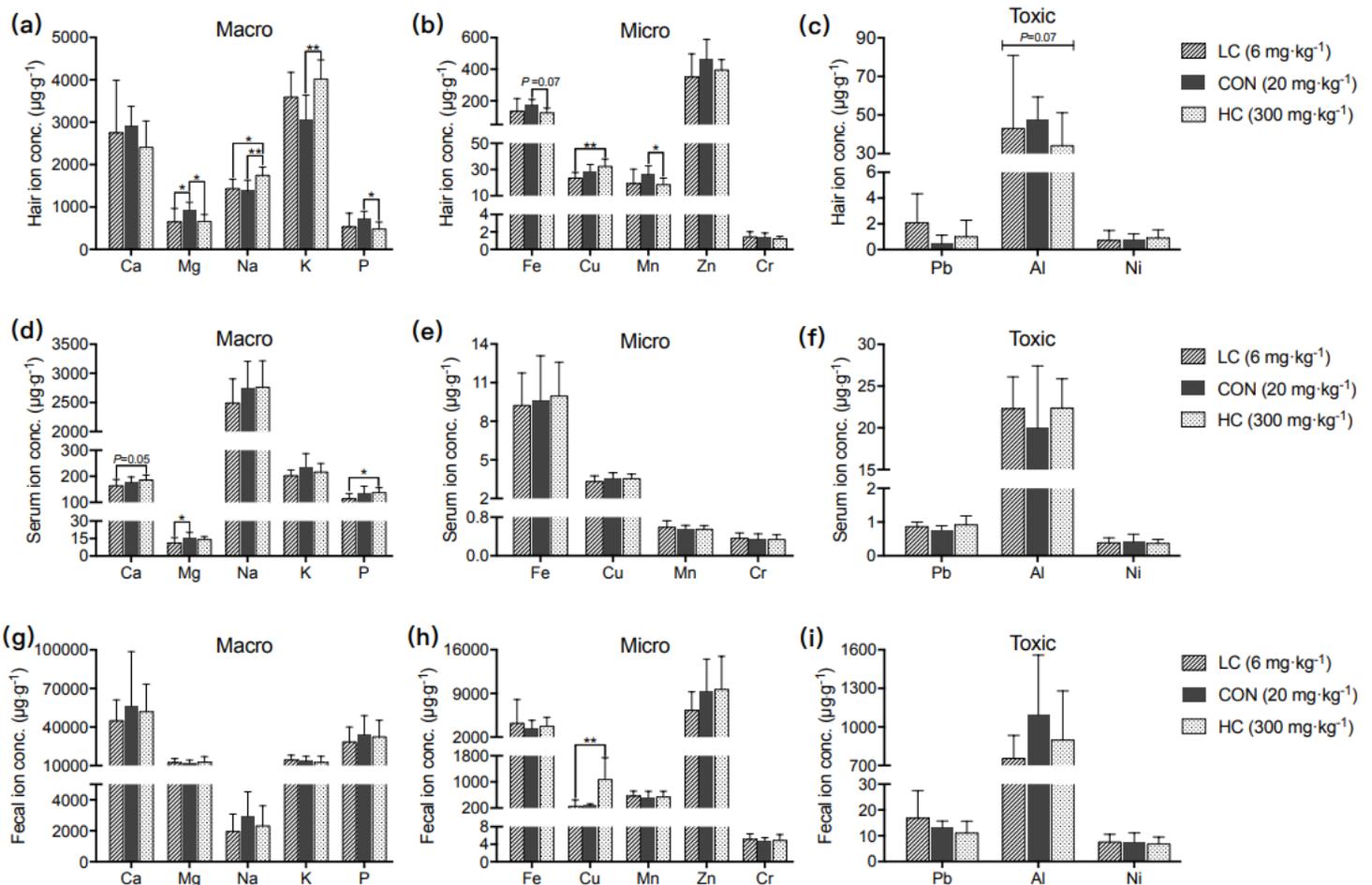


Figure 1

Effect of dietary copper level on hair, serum and fecal ion concentrations in suckling piglets. Over all changes of 13 elements in hair (a, b, c), serum (d, e, f), and feces (g, h, i) among each dietary group.

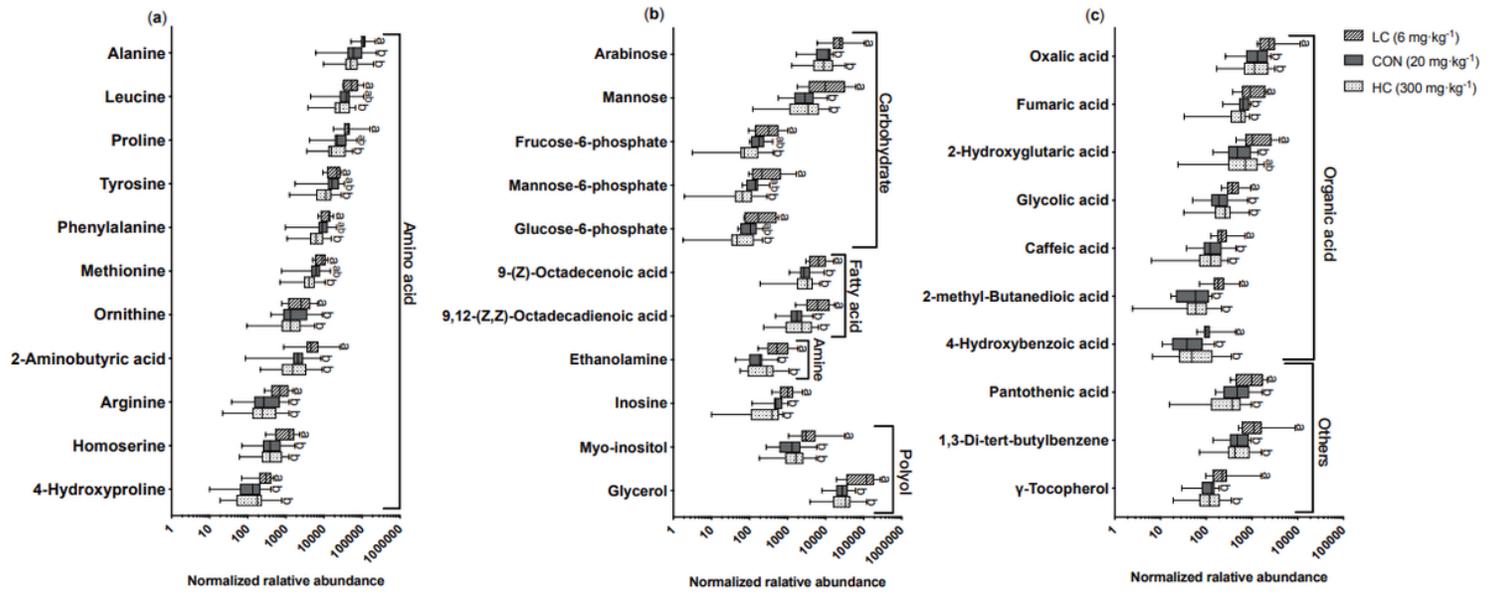


Figure 2

Effect of dietary copper level on the normalized relative abundance of fecal significant metabolites.

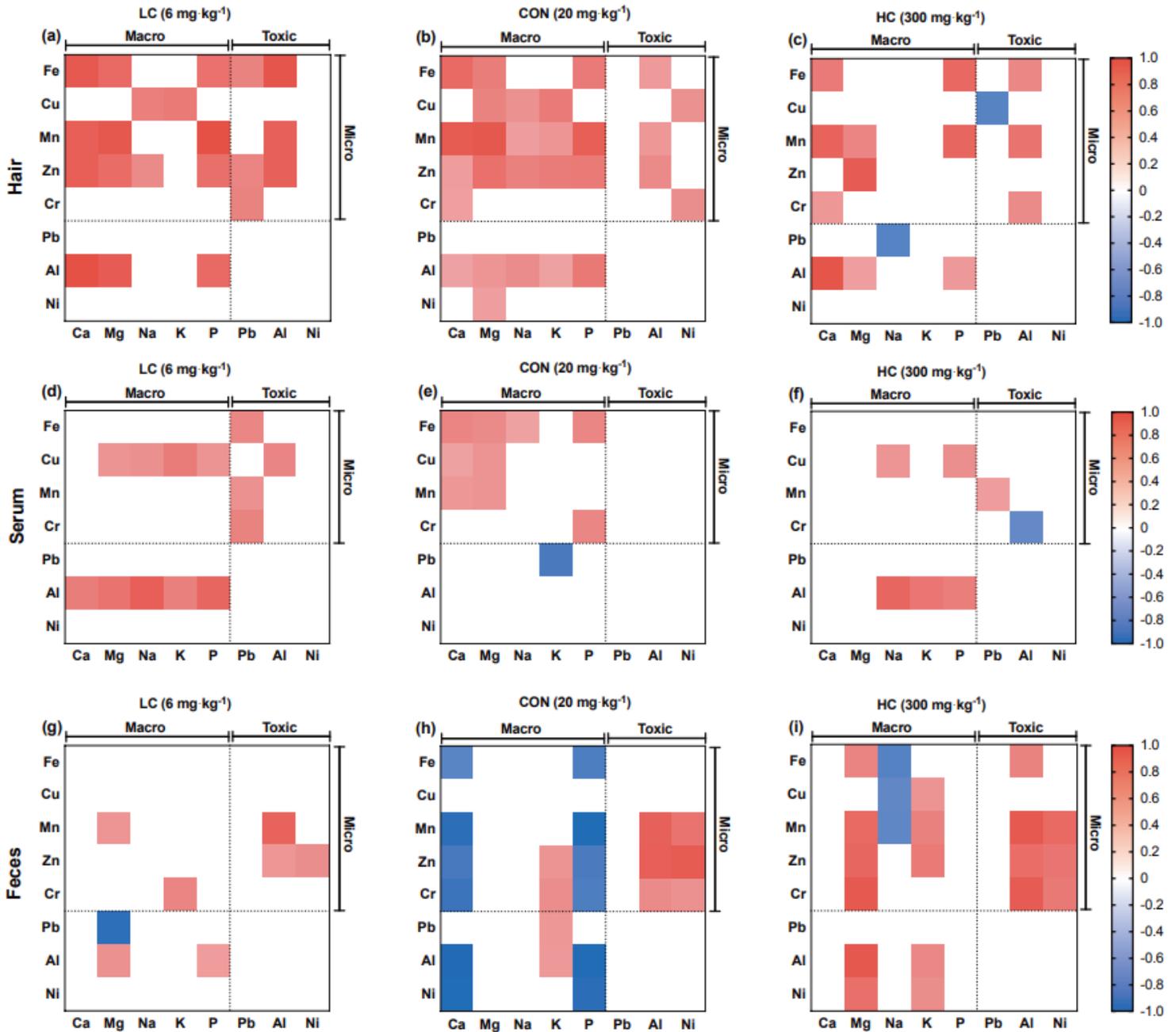


Figure 3

Changes in correlation pattern among hair, serum and fecal elements with different copper level in diet. The macro-micro, macro-toxic and micro-toxic correlation pattern were presented with different dietary copper level (6, 20 and 300 mg·kg⁻¹) in hair (a, b and c), serum (d, e and f), feces (g, h and i). The correlation which existed statistical significant were presented. The red represents a positive correlation ($P < 0.05$), the blue represents a negative correlation ($P < 0.05$), and the white shows that the correlation was not significant ($P > 0.05$).

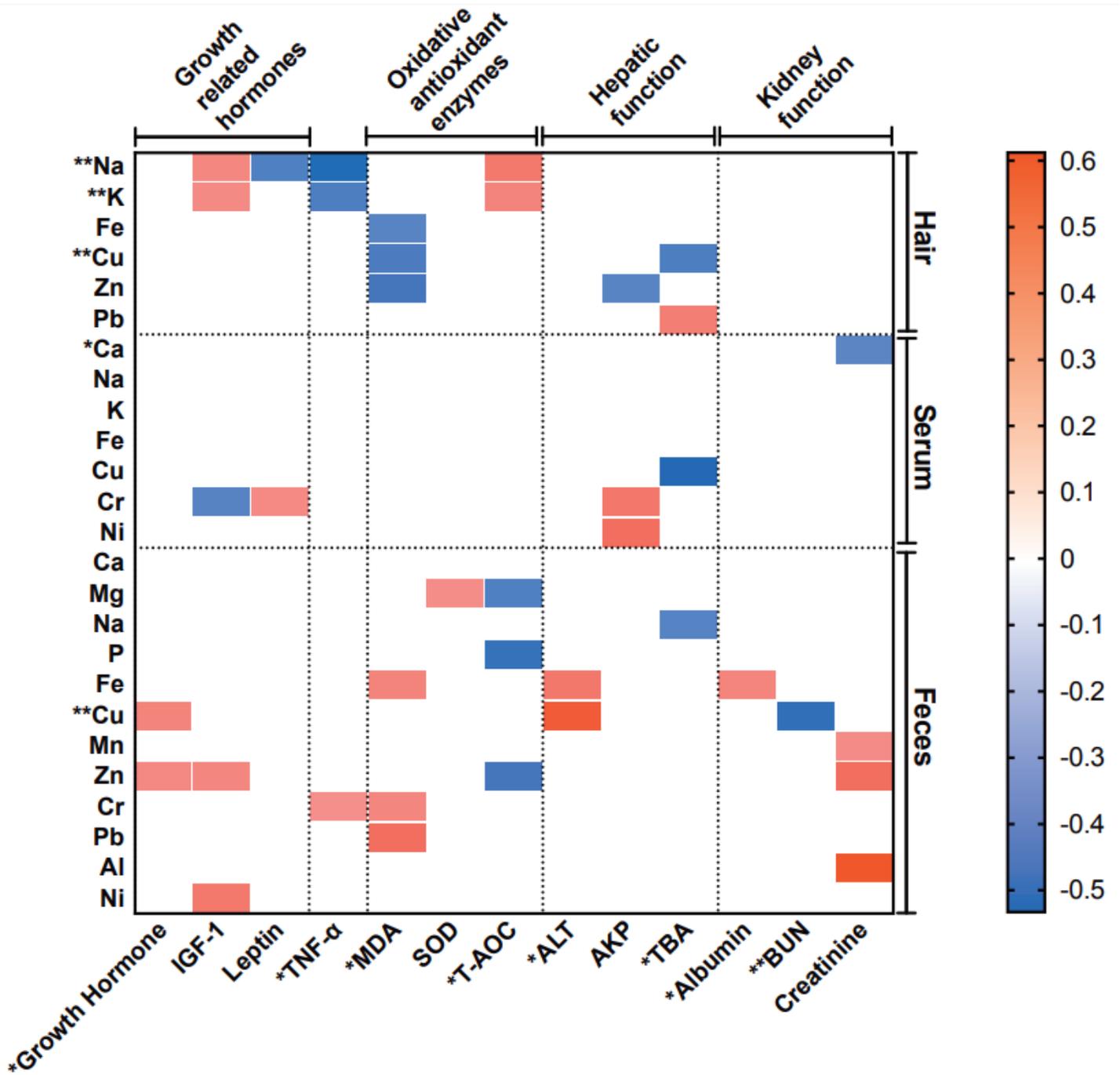


Figure 4

Correlation between ionic profiles and serum biochemical parameters. The correlation which exited statistical significant were presented. The red represents a positive correlation ($P < 0.05$), the blue represents a negative correlation ($P < 0.05$), and the white shows that the correlation was not significant ($P > 0.05$). Significantly different among each copper group, * $P < 0.05$, ** $P < 0.01$.

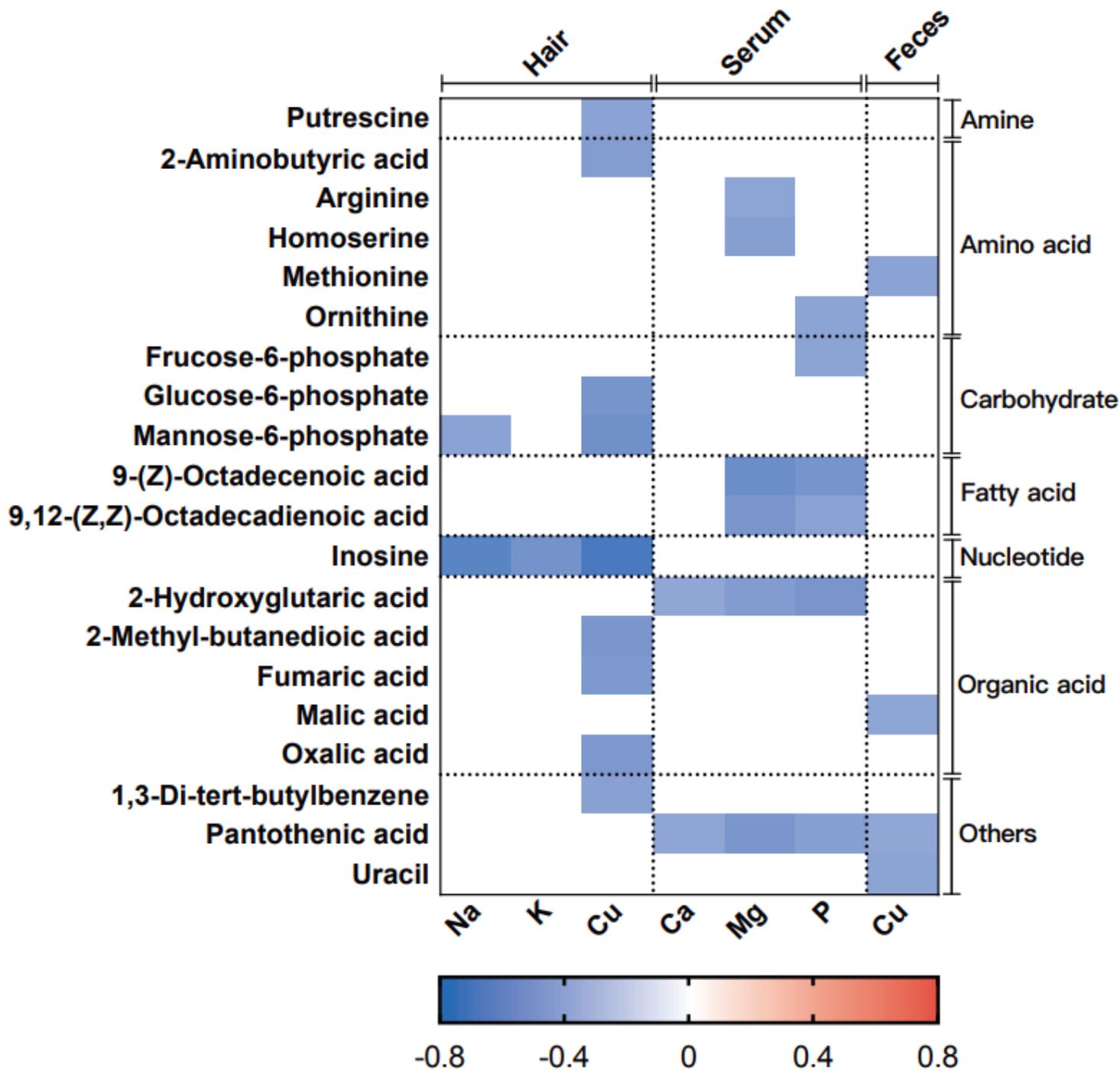


Figure 5

Correlation between ionomic profiles and fecal significant metabolites. The correlation which exited statistical significant were presented. The blue represents a negative correlation ($P < 0.05$), and the white shows that the correlation was not significant ($P > 0.05$).

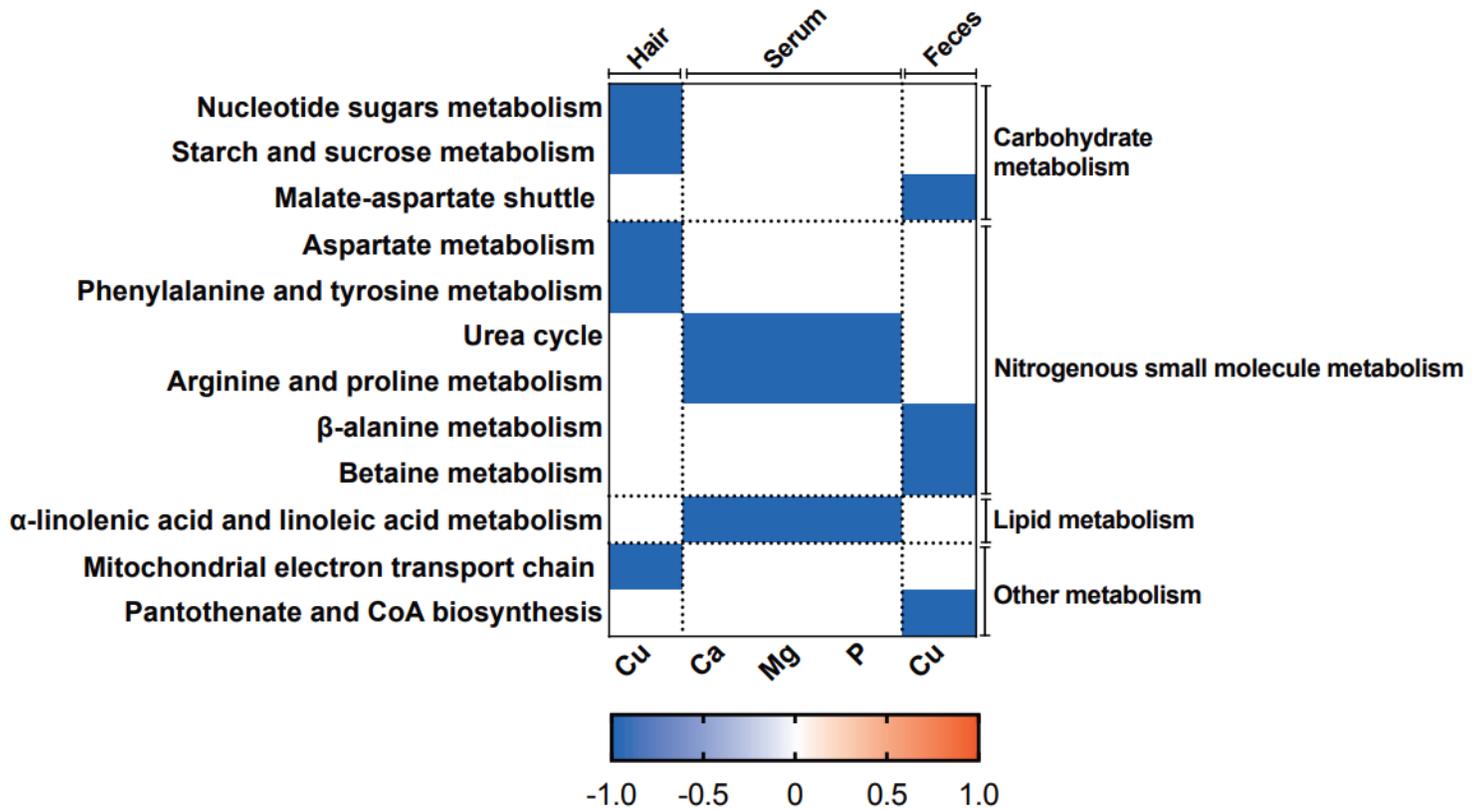


Figure 6

Correlation between ionic profiles and metabolic pathways. The correlation which exited statistical significant were presented. The blue represents a negative correlation ($P < 0.05$), and the white shows that the correlation was not significant ($P > 0.05$).

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