Nursing piglet ionomics-metabolome responses to different dietary copper levels in antibiotic-free creep feed

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Research

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Abstract

**Background:** Ionomics is a novel method to investigate the mechanism underlying the cross-talk and homeostasis of trace elements in animals. Research has not yet determined the composition and distribution of trace elements in piglets and their variations under diverse dietary high-level copper.

**Results:** In this study, the growth performance was calculated from 180 nursing piglets which access to antibiotic-free creep feed including different copper level: 6, 20, and 300 mg·kg\(^{-1}\) Cu (CuSO\(_4\)), and offered *ad libitum* from d 14 until weaning at 40 days of age. In HC (300 mg·kg\(^{-1}\) Cu ) group, the average daily gain (ADG) and average daily feed intake (ADFI) increased during d 14 to 28, but ADG was decreased with extension of feeding time (d 29 to 40). The ionomics profiles (hair, serum, and feces) and the correlations with serum and fecal metabolites were further analyzed. The hair Na, K, Cu, and fecal Cu concentrations were increased in HC group than other groups. The hair Na and K were negatively correlated with serum TNF-\(\alpha\) and fecal inosine, while positively correlated with serum insulin-like growth factors-1 (IGF-1); the hair Cu was negatively correlated with serum malondialdehyde (MDA), total bile acid (TBA) and fecal putrescine, glucose-6-phosphate, fumaric acid; the fecal Cu was positively correlated with serum growth hormone, negatively correlated with fecal methionine, pantothenic acid, and uracil. 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 promoted growth in the short term, and altered the ion balance, further affected the metabolic homeostasis, harm to the health of piglets; dietary 20 mg·kg\(^{-1}\) copper maintain ion homeostasis due to preservation of the interactions between macro and micro elements and suitable to meet the nutritional needs of nursing piglets. These results may benefit people to understand the molecular mechanism of ionomics effects on human and piglets’ health.

**Background**

Copper homeostasis in cells is essential for the normal and healthy growth of mammals, and either excess copper deficiency in cells contributes to abnormalities and diseases [1]. It is also an essential trace element for pigs and harmful to their health when dietary copper is deficient [2]. Copper supplementation is not novelty to pig nutrition, the US National Research Council (NRC) has suggested dietary Cu requirements for 5 to 25 kg nursery pigs and growing pigs of approximately 3 to 6 mg·kg\(^{-1}\) [3], but producers are now feeding them at higher levels to improve pigs’ health [4], post-weaning diets are routinely included with pharmacological Cu (200-250 mg·kg\(^{-1}\)) because of their marked growth promotion and antimicrobial activity [4-8]. However, according to our previous studies in rats and nursing piglets, high dietary levels of copper are scarcely consumed in the intestine and accumulated in intestinal chyme [9, 10], a remarkable accumulation that generates microbial tolerance and resistance to antimicrobial agents, which are considered a public health concerns [4]. Also, the high-grade dietary copper and its
emitted ions, Cu\textsuperscript{2+}, are known to be one of the most significant pollution-causing metals [1]. It has previously been observed that dietary 6 and 300 mg·kg\textsuperscript{-1} copper affect the digestion and utilization of nutrients in feed by alter the composition of the intestinal microbiota and modulating microbial metabolic pathways [10], which can further affect the health of nursing piglets. Research not yet determined the composition and distribution of trace elements in piglets and their variations under diverse dietary copper levels.

Ionomics is a novel multidisciplinary field that uses high-throughput elemental profiling technologies to investigate the mechanism underlying the cross-talk and homeostasis of trace elements in plants and animals [11]. In recent decades, numerous studies have shown that dyshomeostasis of trace elements may be among the risk factors for many complex diseases, such as diabetes [12], neurodegenerative diseases [13], and cancer [14]. A general procedure for ionome measurement, 1) the biological sample of solid tissues, blood, and hair collection; 2) digestion and denaturation of collection samples; 3) measurement of elements by using high-throughput quantitative technologies, such as inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), etc; 4) after statistical analysis, the construction and changes of elemental correlation analyzed; 5) construction of ionome-based models for classification or prediction of phenotypes of interest [11]. The rapid increase in omic data (such as metabolome, genome, and proteome) and recent advances in ionomics methods have facilitated the investigation of the dynamic relationship between trace elements and health or disease, which has been extensively investigated [11]. Nevertheless, it has not been well clarified the molecular mechanisms and the relationship between these elements and the health of the host.

In the current study, hair, blood, and feces samples were obtained from nursing piglets, fed with different copper content and no antibiotics were added. The differential changes were identified and correlation patterns were also analyzed of 13 elements in hair, blood, and feces, the raw data on serum and fecal metabolism from our previous research were used to clarify the underlying relationship between ionomics profiles and metabolism of nursing piglets. To our knowledge, this is the first study about the ionomics-metabolome response to different copper levels of piglets.

**Results**

**Growth Performance**

During phase 1 (d 14 to 28), compared with the LC or CON groups, the ADG ($P < 0.05$) and ADFI ($P < 0.05$) in the HC group increased (Table 1), with extension of feeding time, the ADG ($P < 0.01$) was decreased in the HC group compared with the LC and CON groups (d 29 to 40). The diarrhea rate in 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 nursing piglets (Mean ± SD)
Cu supplementation, mg·kg\(^{-1}\) diet

| Items                  | LC (6)         | CON (20)       | HC (300)      | SEM  | P-value |
|------------------------|----------------|----------------|---------------|------|---------|
| Phase 1, d 14 to 28    |                |                |               |      |         |
| ADG, g                 | 170.77 ± 30.10\(^b\) | 183.28 ± 34.81\(^{ab}\) | 197.45 ± 18.92\(^a\) | 6.89 | <0.05   |
| ADFI, g                | 49.77 ± 4.23\(^{ab}\) | 45.50 ± 5.50\(^b\) | 62.44 ± 15.23\(^a\) | 2.76 | <0.05   |
| G:F                    | 3.47 ± 0.76    | 4.12 ± 1.11    | 3.29 ± 0.77   | 0.22 | 0.26    |
| Phase 2, d 29 to 40    |                |                |               |      |         |
| ADG, g                 | 202.95 ± 26.11\(^a\) | 214.92 ± 16.30\(^a\) | 163.68 ± 36.74\(^b\) | 8.11 | <0.05   |
| ADFI, g                | 120.06 ± 16.06 | 117.88 ± 18.14 | 118.21 ± 21.46 | 4.14 | 0.98    |
| G:F                    | 1.73 ± 0.41    | 1.86 ± 0.29    | 1.45 ± 0.51   | 0.10 | 0.26    |
| Overall, d 14 to 40    |                |                |               |      |         |
| ADG, g                 | 185.92 ± 25.98 | 198.02 ± 20.53 | 183.36 ± 13.28 | 2.76 | 0.44    |
| ADFI, g                | 74.28 ± 4.61   | 72.92 ± 9.03   | 81.30 ± 13.45 | 2.33 | 0.31    |
| G:F                    | 2.51 ± 0.39    | 2.76 ± 0.53    | 2.31 ± 0.45   | 0.11 | 0.27    |
| Diarrhea rate, %       | 8.74 ± 3.99\(^a\) | 6.55 ± 5.37\(^{ab}\) | 2.22 ± 1.18\(^b\) | 1.09 | <0.05   |

\(^{a,b}\) Values within a row without a common superscript letter are significantly different (\(P<0.05\))

**Ionomics profiles**

Thirteen elements concentrations in hair, serum, and feces were presented in Fig. 1. 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 (Fig. 1a-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 (Fig. 1d). In feces, Cu (\(P<0.01\)) concentration increased in the HC group compared with the LC group (Fig. 1h).

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

Correlation patterns among elements at different dietary copper levels are presented in Fig. 2. In hair, the number of correlations in the macro-micro category (upper-left, Fig. 2a-c) in the CON group was more than other groups. Compared with the CON group, the relevance of Cu = f(Mg), Mn = f(Na, K), and Zn = f(K) were lost in both the LC and HC groups. The relevance of Fe = f(Mg), Cu = f(Na, K), Mn = f(Na, K), Zn = f(K) were lost in both the LC and HC groups. The relevance of Fe = f(Mg), Cu = f(Na, K), Mn = f(Na, K), Zn = f(K) were lost in both the LC and HC groups.
f(Na, K), and Zn = f(Ca, P) were lost in the HC group. In the toxic-micro and toxic-macro categories (upper-right and bottom-left, Fig. 2a-c), Ni = f(Cr, Cu) and Al = f(Na, K) were lost 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, Fig. 2d-f), Fe = f(Ca, Mg, Na, P), Cu = f(Ca), Mn = f(Ca, Mg) and Cr = f(P) were lost, while Cu = f(Na, P) appeared in both the LC and HC groups. No significantly positive correlations were observed between toxic and micro or macro elements in the CON group. In feces, with dietary copper levels increased from 20 to 300 mg·kg\(^{-1}\), the negative correlations of Ca and P with most micro and toxic elements were lost, while the positive correlation of Mg and K were appeared (Fig. 2g-i).

**Correlation between ionomics profiles and serum biochemical parameters**

The correlation between ionomics profiles and serum biochemical parameters were presented in Fig. 3. GH was positively correlated with fecal Cu and Zn (\(P < 0.05\)); serum TNF-\(\alpha\) 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 ionomics profiles and fecal significant metabolites**

The correlation between ionomics profiles and fecal significant metabolites were presented in Fig. 4. 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 (see Additional file1, Table S3) which correlated with ionomics profiles were used for further metabolic pathways and metabolite set enrichment analysis (Fig. 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 \(\alpha\)-linolenic acid and linoleic acid metabolism pathways; fecal Cu was negatively correlated with \(\beta\)-alanine metabolism, betaine metabolism, malate-aspartate shuttle, and pantothenate and coenzyme A (CoA) biosynthesis pathways.

**Discussion**
Copper plays an vital role in the synthesis of hemoglobin and the activation of several oxidative enzymes necessary for normal metabolism of pigs[7]. Several studies have revealed that the positive effects of high-grade copper in the feed of piglets are mainly growth promotion [15, 16] and antibacterial activity [4], higher nutritional levels of Cu (as CuSO₄) at concentrations of 100 to 250 mg·kg⁻¹ improved growth performance in young pigs [4, 17]. In our study, LC (6 mg·kg⁻¹ Cu) seems unable to meet the nutritional needs of nursing piglets when antibiotics are withdrawn from creep feed because the G:F value decreased compared with the CON group (20 mg·kg⁻¹ Cu), and the diarrhea rate significantly increased compared with the HC group (300 mg·kg⁻¹ Cu) group. When piglets were fed a 20 mg·kg⁻¹ Cu diet, they tended to gain more and eat less among the three groups across the entire experimental period, which would be adequate to meet the nursing piglets’ requirements. We also observed that 300 mg·kg⁻¹ of dietary Cu enhanced ADG and ADFI during days 14 to 28, but ADG and the G:F value decreased during days 29 to 40. Taken together, these results suggest that high levels of dietary copper can promote short-term growth.

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 [11, 18], hair samples have demonstrated long-term nutritional status compared to biofluids, which often serve as important indicators for mineral content monitoring and toxic metal accumulation in human [19], hair mineral analysis has become an interesting diagnostic tool in the assessment of health and nutritional status [20, 21]. 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 than in the HC group, and these elements share the same uptake mechanism (transferrin), and Fe, Mn, and Al are related to inflammation in humans and animals [22], corroborated by an observed increase in TNF-α in the CON group (see Additional file1, Table S2). 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, e); further, the absorption, utilization, and excretion of many trace elements in animals are greatly affected by other trace elements [23], so these results suggest that dietary 20 mg·kg⁻¹ Cu can maintain homeostasis in piglets due to maintenance of the interactions between macro and micro elements [2]. Correlations between hair macro (Na, K) and micro (Cu, Mn, Zn) elements appear in the HC group (Fig. 2c). 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 [2], suggesting that a dietary dose of 300 mg·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 [22], and marginal or severe element imbalances can be considered risk factors for several diseases [24]. These results suggest that a dietary dose of 300 mg·kg⁻¹ Cu might have adverse effects on nursing piglets’ health.
To understand the relationship between the ion balance and nursing piglets’ health status, correlations between ionomics profiles and serum biochemical parameters were analyzed (Fig. 3). Our previous works on serum biochemical parameters suggested that high dietary levels of copper may have a potential anti-inflammatory effect when antibiotics were withdrawn from the diet, which also had an obvious effect on the antioxidative status of nursing piglets. However, the liver dysfunction might occur in piglets when they fed with 6 and 300 mg·kg$^{-1}$ Cu in diet [10]. 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 mg·kg$^{-1}$ of dietary Cu were 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 [25], and 300 mg·kg$^{-1}$ of dietary Cu may affect the absorption of Fe and further damage the hepatic function, inducing oxidative stress in nursing 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 mg·kg$^{-1}$ of dietary Cu affect the hepatic function and cause oxidative damage [26]. Dietary Cu at a concentration of 300 mg·kg$^{-1}$ increased GH in nursing piglets was demonstrated by the correlation between fecal Cu and GH. The concentrations of Zn in hair and feces were positively correlated with MDA, T-AOC, and creatinine. Zn has the function of stabilizing cell membrane structure and protecting free radicals from oxidative damage [27, 28], and participates in the regulation of liver function and urea production. In general, high-level dietary copper affected the ionomics profiles, which adversely affects the health of nursing piglets.

As we know, fecal metabolites reflect the final status of animal digestion, absorption, and metabolism of feed nutrients [10]. Our previous analysis of significant fecal metabolites showed that the capacity of dietary monosaccharide and protein absorption decreased, and the level of organic acids was increased in nursing piglets fed with 6 mg·kg$^{-1}$ Cu diet (see Additional file1, Fig. S1), these suggest that 6 mg·kg$^{-1}$ Cu supplementation harms the health of piglets when antibiotic withdrawn from the feed [10]. In this study, we are concerned about changes in the composition of elements in hair, which reflect the body’s metabolism changes [24]. 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. 4), consistent with the result that hair Na and K were negatively correlated with serum TNF-α (Fig. 3), due to the inosine is a purine metabolite and has a systemic anti-inflammatory effect [29]. The hair Cu was significantly increased in the HC group than the LC group and negatively correlated with phenylalanine and tyrosine metabolism pathways (Fig. 5), dopamine β-hydroxylase (DBH) and phenylalanine hydrolase are two key enzymes in these pathways which its cofactor is copper [30, 31]. The hair Cu was negatively correlated with the mitochondrial electron transport chain pathway (Fig. 5). Various enzymes in this pathway use copper as a cofactor, such as a cytochrome c oxidase and nicotinamide adenine dinucleotide (NADH) dehydrogenase [30-32]. The activity of the electron transport chain is related to the generation of reactive oxygen species (ROS) and the body’s redox state [33, 34]. In this study, dietary 300 mg·kg$^{-1}$ Cu enhanced the 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 the 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 [35, 36], the process by which betaine provides methyl groups is mainly catalyzed by betaine homocysteine transferase (BHMT), a cytoplasmic enzyme that relies on zinc activation [37], due to the antagonism between copper and zinc [2], suggested that high-level dietary 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 [38, 39], these suggested that 300 mg·kg\(^{-1}\) Cu inhibited the methyl supply capacity of betaine and further affected protein biosynthesis and the health of nursing piglets. Our previous study found that the level of pantothenic acid was decreased in the HC group [10]. The enrichment analysis in this study showed that the fecal Cu was negatively correlated with pantothenate and CoA biosynthesis pathway, pantothenic acid plays an important role in the process of decomposing carbohydrates, fatty acids, and amino acids to produce energy [40]. These results suggest that dietary high-level copper affected the digestion and absorption of diets by piglets, which affected the biosynthesis of pantothenic acid and CoA, further affecting carbohydrate, fat, amino acid, and energy metabolism of nursing piglets, leading to changes in metabolic homeostasis.

**Conclusions**

In conclusion, 300 mg·kg\(^{-1}\) of dietary copper promoted growth in the short term but exhibited a lack of 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. Six mg·kg\(^{-1}\) of dietary Cu seems unable to meet the nutritional needs of suckling piglets. Twenty mg·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. It seems suitable for meeting the needs and maintaining the health of suckling piglets. Our results may benefit people to understand the molecular mechanism of ionomics effects on piglets’ metabolism.

**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 into three groups (6 litters/group, ten piglets per litter): (1) low copper diet (LC, 6 mg·kg\(^{-1}\)) containing no supplemental Cu; (2) control diet (CON, 20 mg·kg\(^{-1}\)); or (3) high copper diet (HC, 300 mg·kg\(^{-1}\)), with supplementation utilizing copper sulfate (CuSO\(_4\)). 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 within 72 h after farrowing. 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 [3] (see Additional file1, 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 were hindered from having access and eating the sow feed [10].

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 was 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 average daily gain (ADG) and average daily feed intake (ADFI). The diarrhea rate of piglets was recorded daily and calculated as follows: Diarrhea rate (%) = the number of pigs with diarrhea × diarrhea days / (the total number of pigs × experiment days) × 100%, which the “number of pigs with diarrhea” was defined as the number of piglets with diarrhea was observed each day [41].

Three litters were chosen within each treatment at 40 days. All samples were collected from four piglets/litter, selected based on average body weight (half male and female). Hair samples of the head, back, and buttocks of the piglets were collected and mixed; Blood samples were collected from the anterior vena cava of piglets and stored in glass tubes with no anticoagulant and were allowed to clot at 4 °C before the serum harvest by centrifugation (15 min at 3500 rpm); serum, hair and fecal samples were stored at-80 °C for subsequent analysis [9].

**Ionomics 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 were brought to constant volume with double distilled-deionized water [9, 42]. The standard liquid of Ca, Mg, Na, K, and P (1000 µg·mL⁻¹) were mixed to prepare a 5 mL mixed standards with 0.5 mol·L⁻¹ HNO₃. Take 0.5 mL mixed standard of Fe, Cu, Mn, Zn and Cr (1000 µg·mL⁻¹), constant volume to 5 mL secondary mother liquor (100 µg·mL⁻¹), then using secondary mother liquor prepare 10 mL standard with 0.5 mol·L⁻¹ HNO₃. Take 0.5 mL mixed standard of Pb, Al, and Ni (1000 µg·mL⁻¹), constant volume 2 times and prepare 5 mL secondary mother liquor (10 µg·mL⁻¹), then prepare 10 mL standard with 0.5 mol·L⁻¹ HNO₃. 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 the standard curve, according to the standard curve of the conversion of each element contained in the samples.

**Serum biochemical parameters analysis and the correlation with ionomics profiles**

Serum biochemical parameters were detected for growth hormone (GH), insulin-like growth factors-1 (IGF-1), leptin, and tumor necrosis factor-α (TNF-α) using the enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), other indexes including malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), alanine aminotransferase (ALT), aspartate transaminase (AST), total bile acid (TBA), Albumin, blood urea nitrogen (BUN), and Creatinine content in serum was directly detected by using the commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with UV-VIS spectrophotometer (Thermo, USA) according to the manufacturer's instructions. The analysis of serum biochemical parameters was done, and the results were published [10], the details were shown in Additional file 1 (Table S2), in which the raw data was used to analyze the correlation with ionomics profiles.

**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, Additional materials and methods) [10]. 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 [10]. The correlation between ionomics and significant fecal metabolites was analyzed.

**Statistical analysis**

Growth performance and ionomics profiles were compared among the groups using 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 performed using SPSS Statistics Version 22.0 (https://www.ibm.com/analytics/spss-statistics-software).

**List Of Abbreviations**

ADFI: Average daily feed intake; ADG: Average daily gain; ALT: Alanine aminotransferase; AST: Aspartate transaminase; BHMT: Betaine homocysteine transferase; BUN: Blood urea nitrogen; DHB: Dopamine β-hydroxylase; ELISA: Enzyme-linked immunosorbent assay; GH: Growth hormone; GR: Guaranteed reagent; ICP-MS: Inductively coupled plasma mass spectrometry; ICP-OES: Inductively coupled plasma optical emission spectrometry; IGF-1: Insulin-like growth factors-1; MDA: Malondialdehyde; NADH: Nicotinamide
adenine dinucleotide; SOD: Superoxide dismutase; T-AOC: Total antioxidant capacity; TBA: Total bile acid; TNF-α: Tumor necrosis factor-α; VIP: Variable important projection.

**Declarations**

**Ethics approval and consent to participate**

We did not use human subjects/samples in this study. 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**

The datasets used during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The following authors F.Z., W.Z., Y.X. and W.Y. declare no conflict of interest.

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**Author Contributions**

Conceived and designed the experiments: F.Z., W.Z., W.Y. Performed the experiments: F.Z., Y.X. Statistical data analyzed: F.Z., Y.X. Wrote and revised the manuscript: F.Z., W.Y. All authors have read and agreed to the published version of the manuscript.

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**Figures**

**Figure 1**

Effect of dietary copper levels on hair, serum and fecal ion concentrations in suckling piglets. Overall changes of 13 elements in hair (a, b, c), serum (d, e, f), and feces (g, h, i) among each dietary group. * denote P < 0.05, ** denote P < 0.01.
Figure 2

Changes in correlation pattern among hair, serum and fecal elements with different copper levels in diet. The macro-micro, macro-toxic and micro-toxic correlation pattern were presented with different dietary copper levels (6, 20 and 300 mg·kg⁻¹) in hair (a, b and c), serum (d, e and f), and feces (g, h and i). 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).
Figure 3

Correlation between ionomics 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, * denote \( P < 0.05 \), ** denote \( P < 0.01 \).
Figure 4

Correlation between ionomics 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 5

Correlation between ionomics 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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