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Dietary copper modulates intestinal digestive function and gut microbiota in the Chinese mitten crab (*Eriocheir sinensis*)

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Abstract

Copper (Cu) is an essential trace element for aquatic animals, but its role in regulating intestinal digestive function and microbiota-associated nutrient processing, especially through host‑microbiota interactions, remains poorly understood. This study evaluated the dose-dependent effects of diets supplemented with 0, 18, 28, and 38 mg·kg^{-1} $CuSO_4 \cdot 5H_2O$, corresponding to total dietary Cu concentrations of 9.02, 26.36, 36.69, and 47.72 mg·kg^{-1}, respectively, on intestinal digestive enzyme activities, nutrient transport-related gene expression, and gut microbiota in *Eriocheir sinensis*. The Cu28

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diet (total dietary Cu = 36.69 mg·kg⁻¹) increased the activities of intestinal α-amylase, trypsin, and lipase, while excessive Cu supplementation (38 mg·kg⁻¹) weakened these responses. In parallel, the key transport-related genes (*slc1a2*, *slc7a5*, and *slc7a8*) were up-regulated in the Cu28 group, but down-regulated under high copper conditions. Dietary Cu altered the gut microbiota in a dose-dependent manner. The Cu28 group was characterized by increased abundances of *Bacteroides*-associated and *Candidatus Bacilloplasma*-associated ASVs, whereas excessive Cu enriched *Candidatus Hepatoplasma* and altered the composition of discriminative bacterial taxa. Consistently, the Cu28 group showed higher network complexity and connectivity, indicating increased network connectivity. Functional prediction suggested that pathways related to bacterial motility, chemotaxis, and biofilm formation were enriched in the Cu28 group, while pathways associated with ABC transporters and quorum sensing were reduced under high copper conditions. In summary, dietary Cu may influence intestinal nutrient processing by coordinating digestive enzyme activities, transport-related gene expression, and changes in gut microbiota. The Cu28 diet was associated with improved intestinal physiological status and microbial stability, whereas excessive Cu showed the opposite trend. This study provides new insights into the comprehensive role of dietary copper in coordinating host‐microbiota interactions to regulate intestinal nutrient-processing capacity in aquatic animals.

Keywords: Copper, intestinal function, nutrient processing, gut microbiota, *Eriocheir sinensis*

1. Introduction

The Chinese mitten crab (*Eriocheir sinensis*) is one of the most important freshwater aquaculture species in China, which has a great contribution to aquaculture production [1, 2]. With the increasing intensification of aquaculture systems, there is a growing need for precise nutritional strategies to maintain animal health and improve production efficiency[3, 4]. Although the requirements of *E. sinensis* for

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macronutrients such as proteins and amino acids have been extensively studied [5], the roles of trace elements in regulating physiological functions and intestinal health are still poorly understood.

Copper (Cu) is an essential trace element for aquatic animals. As a cofactor for enzymes involved in energy metabolism, redox regulation, and antioxidant defense, copper plays a vital role in maintaining growth performance and physiological homeostasis, whereas Cu deficiency impairs nutrient absorption and metabolic processes [6, 7]. In the natural water environments, the concentration of Cu^{2+} is usually lower than 5 $\mu g \cdot L^{-1}$ [8], which is not enough to meet the physiological needs. Since copper cannot be de novo synthesized by aquatic animals, it must be obtained from exogenous sources, mainly through diet [6]. Consequently, the addition of copper to diets has become a common practice in aquaculture. Among them, copper sulfate ($CuSO_4 \cdot 5H_2O$) has become one of the most widely used sources due to its low cost, high solubility, and ease of incorporation into feed formulations [6, 9]. Previous studies have shown that in a relatively wide range (20.78–40.34 $mg \cdot kg^{-1}$), dietary copper supplementation can improve the growth performance and physiological status of *E. sinensis* [10]. However, in different studies, the optimal range is still inconsistent and relatively wide, making it difficult to define a precise nutritional requirement [10-12]. Importantly, the safety range of copper is narrow, and excessive Cu can cause toxic effects, affect growth performance and feed conversion, and pose risks to both organismal health and environmental safety [12]. Therefore, determining a "safe-optimal" range of dietary Cu to maximize its nutritional benefits without causing Cu overload remains a key challenge for aquaculture nutrition [6].

The intestine serves as the main site for nutrient digestion and absorption, the intestinal tract is one of the most important interfaces in response to dietary Cu, thereby mediating its biological effects [13, 14]. The efficient nutrient processing depends on the coordinated processes of epithelial digestion and transmembrane transport [15]. There is increasing evidence that dietary copper can affect epithelial

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absorption by affecting intestinal morphology and barrier integrity, thereby supporting effective nutrient absorption [16, 17] These findings suggest that Cu plays an important role in maintaining the structural and functional basis of intestinal absorption. However, existing studies have primarily focused on morphological and general health indicators, whereas the regulatory role of Cu in epithelial nutrient transport processes, remains poorly characterized, especially in crustaceans.

Concurrently, the intestine harbors a complex microbial community that substantially contributes to host nutrient processing by degrading complex dietary polysaccharides, thereby complementing host digestive functions and enhancing nutrient acquisition efficiency [18-20]. Dietary components are recognized as key drivers of gut microbiota composition and function in aquatic animals [21, 22]. In addition to metabolic activity, motility and chemotaxis, which are ubiquitous properties of bacteria, enable them to respond to nutrient gradients and consequently influence substrate utilization within the intestinal environment [23, 24]. Interactions among microbial taxa further shape the efficiency and stability of substrate degradation, and a well-connected microbial network promotes coordinated and sequential utilization of dietary substrates [25, 26]. Despite these advances, previous associated studies have primarily examined the effects of Cu at either the crab physiological level or the microbial compositional level, with limited integration of these two systems. This separation limits the understanding of how dietary Cu regulates the host–microbiota axis to regulate nutrient acquisition and metabolic efficiency. Therefore, there is still a lack of a comprehensive framework linking dietary copper, microbial dynamics and intestinal function regulation.

In order to address these gaps, we evaluated the effects of dietary gradient copper (0, 18, 28, and 38 $mg \cdot kg^{-1}$ $CuSO_4 \cdot 5H_2O$) on intestinal digestive enzyme activities, nutrient absorption-related gene expression, and gut microbial communities of *E. sinensis*. By integrating host physiological indicators and

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microbial analysis based on 16S rRNA, this study provides a comprehensive perspective on the role of dietary copper in regulating intestinal digestive function and microbiota-associated nutrient processing.

2. Materials and Methods

2.1 Experimental design and diets

Four isonitrogenous and isolipidic diets were formulated, and there were only differences in feed copper levels. $CuSO_4 \cdot 5H_2O$ (purity ≥ 99%; Wanqing Chemical Glass Instrument Co., Ltd., Nanjing, China) was used as the copper source, and four experimental groups (Con, Cu18, Cu28, Cu38) were generated by adding 0, 18, 28, and 38 $mg \cdot kg^{-1}$ $CuSO_4 \cdot 5H_2O$. The detailed diet composition is shown in Table S1.

2.2 Crab culture and ethics statement

Juvenile *E. sinensis* with an initial body weight of 26.45 ± 0.85 g were obtained from the Aquatic Teaching Base of Nanjing Agricultural University (Jiangsu, China). After a 7-day acclimation period, the crabs were randomly assigned to 12 cement ponds (1.0 × 1.0 × 0.8 m), with 10 crabs per pond and 3 replicates per treatment. The feeding trial lasted for 8 weeks, during which 5% of body weight was fed once a day. The water quality parameters were maintained as follows: dissolved oxygen 6.0–8.0 $mg \cdot L^{-1}$, temperature 24–28°C, pH 7.0–8.0, ammonia nitrogen < 0.05 $mg \cdot L^{-1}$, and baseline copper concentration < 0.0015 $mg \cdot L^{-1}$.

All procedures complied with the guidelines for experimental animal care in Jiangsu Province (Jiangsu Provincial Government Order No. 45, released 19 August 2008, effective from 1 October 2008) and were approved by the Animal Care and Use Committee of Nanjing Agriculture University (approval number SYXK20210086).

2.3 Sample collection

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At the end of the feeding trial, crabs were sampled after fasting for 24 h. Intestinal tissues and contents were aseptically collected under sterile conditions on ice. Samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA and DNA extraction.

2.4 Digestive enzyme activities

Intestinal tissues were homogenized (1:9, *v/w*) in ice-cooled saline, centrifuged at 2500 × g for 15 min at 4° C, and supernatants were collected. The activities of amylase, lipase, and trypsin were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), n = 6.

2.5 RNA extraction and qPCR

Total RNA was extracted from intestinal tissues using TRIzol reagent (Invitrogen, CA, USA). RNA concentration and purity were determined by spectrophotometry, and samples with A260/A280 ratios of 1.9–2.1 and A260/A230 ratios above 2.0 were used for subsequent analyses. RNA integrity was preliminarily assessed by agarose gel electrophoresis before cDNA synthesis (Fig. S4). First-strand cDNA was synthesized using the Evo M-MLV RT Premix (Accurate Biology, Hunan, China). qPCR was performed on a CFX Connect™ (Bio-Rad, USA) using the SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biology). Gene-specific primers were synthesized by Sangon (Table S2). Primer amplification efficiencies were determined using standard curves generated from serially diluted cDNA templates (Fig. S6), and melt curve analysis was performed to confirm amplification specificity (Fig. S5). The ubiquitin/ribosomal S27 fusion gene served as the internal reference, and its stability was evaluated based on the consistency of Cq values among treatments (Table S4). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [27], n = 6.

2.6 Intestinal microbiota sequencing and bioinformatics analysis

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Microbial genomic DNA was extracted from intestinal samples using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, USA). DNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometry. The V3‑V4 region of the bacterial 16S rRNA gene was amplified using universal primers 338F and 806R. PCR products were purified, quantified, and sequenced on the Illumina NovaSeq-PE250 platform (Illumina, USA) by Biozeron Biotechnology Co., Ltd. (Shanghai, China), n = 6.

QIIME2 was used to perform quality filtering, denoising, and merging of the original reads to generate amplicon variants (ASVs), which were then subjected to taxonomic annotation. Shannon and Simpson indexes were used to evaluate the Alpha diversity of intestinal bacterial community. Beta diversity was assessed by principal coordinate analysis (PCoA) based on Bray-Curtis distances, and the difference between groups was tested by permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, using the adonis2 function in the R package vegan. Statistical significance was determined at $P < 0.05$. The relative abundance of intestinal microbes at the phylum and genus levels was calculated. Circos plots, linear discriminant analysis effect size (Lefse) analysis and Tax4Fun2 (v1.1.5) functional prediction were used to evaluate community composition, differential groups, and potential functional spectrum, respectively [28]. Genus LEfSe using the “trans_diff” function in the "microeco" R package (v2.2.0). Taxa with a Kruskal-Wallis $P < 0.05$ and a logarithmic LDA score threshold of 2.0 were considered significantly differentially abundant [29]. Further, a co-occurrence network was constructed based on Spearman’s correlation coefficient between dominant genera, and the correlation coefficient satisfying $|r| > 0.8$ and $P < 0.05$ was retained for analysis. Then, the network topology attributes, including the number of nodes, the number of edges, and the proportion of positive and negative correlations, were calculated. Mfuzz (v2.60.0) cluster analysis was performed in R (v4.3.1) to determine the dynamic pattern of ASVs under different dietary copper levels [30].

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2.7 Statistical analysis

All data are presented as mean ± SEM. Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Differences among groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison test when the assumptions of normality and homogeneity of variance were met. Statistical significance was set at $P < 0.05$. Microbial analyses were performed in QIIME2, including diversity analysis, PCoA, PERMANOVA, LEfSe (LDA > 2.0), and Spearman-based network construction.

3. Results

3.1 Intestinal digestive enzyme activities under different copper levels

Compared with the control group, intestinal α-amylase activity increased significantly in the Cu28 group ($P < 0.05$), but declined at the higher supplementation level (Cu38) and was significantly lower than that in the Cu28 group (Fig. 1A). Trypsin and lipase activities exhibited a similar pattern. Both enzymes were significantly elevated in the Cu18 and Cu28 groups compared with the control ($P < 0.05$), whereas no significant differences were observed between the Cu38 and control groups ($P > 0.05$) (Fig. 1B, C).

3.2 Expression of intestinal nutrient transport-related genes

Compared with the control group, the expression levels of *slc1a2*, *slc7a5*, and *slc7a8* were significantly increased in the Cu28 group ($P < 0.05$), but decreased in the Cu38 group ($P < 0.05$) (Fig. 2A-C). A similar trend was observed for *slc17a5*, *slc26a6*, and *slc38a9*, which showed higher expression in the Cu18 and Cu28 groups, with peak levels generally occurring in the Cu28 group. In contrast, their expression in the Cu38 group was significantly lower than that in the other treatments ($P < 0.05$) (Fig. 2D-F). To provide additional physiological context, growth performance data are summarized in Table S3. Briefly, the Cu28 group showed higher weight gain and specific growth rate compared with the control group, while

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excessive $CuSO_4 \cdot 5H_2O$ supplementation did not further improve growth performance. These growth responses were generally consistent with the observed changes in intestinal enzyme activities and nutrient transport-related gene expression.

3.3 Gut microbial diversity and community structure

Rarefaction analysis showed that both ASV richness and the Shannon index approached saturation with increasing sequencing depth, suggesting that sequencing coverage was sufficient to capture the major microbial taxa (Fig. S1). After quality control, a total of 1,761 ASVs were identified across all groups, of which 804 were shared among groups, while each treatment also harbored a number of unique ASVs. Notably, the number of unique ASVs increased with increasing dietary copper concentration (Fig. 3A). PCoA showed that the microbial community was significantly separated after dietary copper supplementation compared with the control group, and PERMANOVA confirmed that these differences were statistically significant ($R^2 = 0.4584$, $P = 0.001$) (Fig. 3B). Alpha diversity analysis further showed that the Shannon and Simpson indexes of the Cu38 group were significantly lower than those of the control group, while no significant differences were detected in the Cu18 and Cu28 groups (Fig. 3C, D).

3.4 Taxonomic composition of the intestinal microbiota

At the phylum level, Bacteroidota, Firmicutes, and Proteobacteria were dominant in each group, and their relative abundances were not significantly different among treatments (Fig. 4A). At the genus level, the microbiota was primarily composed of *Candidatus Bacilloplasma* (7.44 - 17.91%), *Dysgonomonas* (5.47 - 22.99%), Bacteroides (3.00 - 16.38%), *Roseimarinus* (4.54 - 11.34%), and *Candidatus Hepatoplasma* (0.49 - 17.41%) (Fig. 4B). LEfSe analysis revealed different microbial characteristics between treatment groups (Fig. 4C). The control group was characterized by enrichment of *Acinetobacter*, *Parabacteroides*, *Rhodobacter*, and *Bifidobacterium*. On the contrary, *Dysgonomonas*, *Pragia*, and

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Morganella were enriched in the Cu18 group, while *Malaciobacter*, *Vibrio*, and *Pseudomonas* were more abundant in the Cu28 group. The Cu38 group showed enrichment of *Candidatus Hepatoplasma*, *Bacteroides*, *Prevotella*, and *Barnesiella*.

Further comparison showed that there were different response patterns to dietary copper levels. *Dysgonomonas* increased significantly in the Cu18 group, while *Candidatus Hepatoplasma* increased gradually with the increase of copper level, and reached the highest abundance in the Cu38 group. *Bacteroides* remained relatively abundant in the Cu28 and Cu38 groups, while *Roseimarinus* decreased with the increase of copper supplementation (Fig. 4D; Fig. S2). Mfuzz cluster analysis identified six distinct dynamic patterns of ASVs at different copper levels (Fig. 4E-F). Among these, Cluster 2 exhibited a gradual increase from the control to the Cu28 group, and then decreased in the Cu38 group (Fig. 4E). Taxonomic analysis further indicated that Cluster 2 was mainly composed of *Bacteroides* and *Candidatus Bacilloplasma* (Fig. 4F). Cluster 3 decreased significantly after copper supplementation (Fig. 4E). Taxonomic analysis indicated that Cluster 3 included *Acinetobacter*, *Afipia*, *Arenimonas*, and *Pandoraea* (Fig. 4F). Consistent with these patterns, ASVs affiliated with *Bacteroides* (ASV1), *Candidatus Bacilloplasma* (ASV16, ASV21), *Vibrio* (ASV30), and *Macellibacteroides* (ASV41) were more abundant in the Cu28 group but declined in the Cu38 group. In contrast, ASVs related to *Acinetobacter* (ASV35, ASV53), *Afipia* (ASV38), *Arenimonas* (ASV37), and *Pandoraea* (ASV65) decreased following copper supplementation and remained at relatively low levels in both the Cu28 and Cu38 groups (Fig. 4G).

3.5 Functional potential of the gut microbiota under different dietary copper levels

PCoA analysis showed only a slight shift in functional profiles among treatments, with no clear separation between groups (Fig. 5A). At the KEGG pathway level, predicted functions were mainly associated with metabolism (global and overview maps, carbohydrate metabolism, and amino acid

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metabolism) and environmental information processing (membrane transport and signal transduction) (Fig. 5B). Further differential analysis indicated that dietary copper exerted dose-dependent effects on microbial functional pathways. Compared with the control group, the Cu18 group exhibited limited functional alterations, with bacterial chemotaxis being the most notably affected pathway (Fig. 5C), In the Cu28 group, multiple pathways were significantly enriched, including bacterial chemotaxis, flagellar assembly, cell cycle - Caulobacter, and biofilm formation - *Pseudomonas aeruginosa* (Fig. 5D). With increasing copper levels, the functional shifts became more pronounced. In the Cu38 group, pathways related to two-component systems, bacterial chemotaxis, flagellar assembly, and biofilm formation were significantly elevated, whereas pathways related to ABC transporters and quorum sensing were significantly downregulated compared with the control group (Fig. 5E). Consistently, compared with the Cu18 group, ABC transporter pathway was the top downregulated pathway (Fig. 5F). Notably, the downregulation of ABC transporter pathways, also including quorum sensing, were not observed in the other copper-treated groups, but only the Cu38 group (Fig. 5G-H). Across all copper-treated groups, the shared pathways bacterial chemotaxis and flagellar assembly consistently showed increased relative abundance compared with the control group (Fig. 5I-J). Overall, these results indicate that dietary copper reshapes microbial functional potential in a dose-dependent manner, primarily affecting pathways related to motility, environmental sensing, and intercellular interactions.

3.6 Co-occurrence network of the gut microbiota

To further investigate the effects of dietary copper on microbial community interactions, co-occurrence network analysis was performed (Fig. 6A). In the Cu18 group, the number of edges increased to 622, with 97 nodes, indicating a moderate increase in microbial associations (Fig. 6B). It is worth noting that the Cu28 group showed the highest network complexity, with 100 nodes and 950 edges, reflecting a significant

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increase in connectivity and microbial associations (Fig. 6C). In contrast, the connectivity of the Cu38 group (706 edges with 99 nodes) was lower than that of the Cu28 group, but still higher than that of the control group (Fig. 6D). The correlation structure of the networks also shifted in response to dietary copper. In the control group, positive associations predominated (75.54%). However, copper supplementation reduced the proportion of positive correlations while increasing negative correlations. Specifically, in the Cu18, Cu28 and Cu38 groups, the proportion of positive correlation decreased to 62.48 %, 63.05 % and 64.16 %, respectively (Fig.6E), reflecting a shift toward a higher proportion of negative taxon–taxon associations.

4. Discussion

Dietary copper supplementation, especially in the form of copper sulfate ($CuSO_4 \cdot 5H_2O$), has become a common practice in aquaculture [31-33]; however, due to its narrow safety range, its optimal dietary range is still controversial. In the present study, the Cu28 diet significantly enhanced the activities of digestive enzymes and the expression of transport-related genes in the intestine of *E. sinensis* (Fig. 1), indicating that this supplementation level was favorable for supporting intestinal digestive and transport-related responses. This finding is consistent with previous reports in aquatic animals, and further narrows the effective range associated with beneficial intestinal responses [10]. In crustaceans, digestion depends on a synergistic enzyme system dominated by proteases, amylases, and lipases, which together determine digestion efficiency [34]. Trypsin is mainly responsible for the hydrolysis of proteins and the activation of digestive enzymes, whereas amylase and lipase are involved in the digestion of carbohydrates and lipids, respectively [35, 36]. In this study, the Cu28 diet significantly increased the activities of α-amylase, protease, and lipase, which was consistent with the results in fish [37, 38]. This stimulating effect may be related to the important role of copper as a trace element in maintaining intestinal physiological

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homeostasis and supporting enzyme activity, supporting intestinal nutrient-processing capacity. These results suggest that appropriate copper levels can improve the digestive efficiency of *E. sinensis*, thereby supporting intestinal nutrient processing. Growth performance data presented in Table S3 and a broken-line regression analysis (Fig. S2) provided additional physiological context for these intestinal responses, as the Cu28 group showed relatively improved weight gain and specific growth rate, whereas excessive $CuSO_4 \cdot 5H_2O$ supplementation did not further improve growth performance.

Efficient intestinal nutrient processing further depends on the solute carrier (SLC) transporter-mediated epithelial transport process, regulating the uptake of amino acids and other nutrients through the intestinal epithelium [39]. Our results show that under moderate copper supplementation, key amino acid transporters, including *slc1a2*, *slc7a5* and *slc7a8*, were up-regulated (Fig. 2A‑C), indicating that copper availability directly affects intestinal transport capacity. Since SLC1A2, SLC7A5 and SLC7A8 are closely related to nutritional perception and metabolic regulation, these changes may reflect changes in anabolism [40-42]. In addition, other SLC members related to cell homeostasis [43, 44], such as *slc17a5*, *slc26a6* and *slc38a9*, also showed similar down-regulation under high copper conditions (Fig. 2D‑F), indicating that excessive copper may be associated with changes in epithelial stability and intracellular metabolic balance. Although the direct evidence linking dietary copper to SLC transporter expression is still limited, previous studies have shown that copper-induced oxidative stress and intestinal damage may impair epithelial transport capacity in aquatic animals, thereby potentially affecting nutrient absorption [45]. In summary, these findings highlight the role of copper in regulating epithelial nutrient transport, thereby contributing to intestinal nutrient-processing potential.

The intestinal microbiota is an important component associated with nutrient digestion, absorption, and growth [36, 46]. In the present study, dietary copper supplementation significantly changed the intestinal

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microbial community (Fig.3), which is consistent with the previously reported dietary composition shaping the intestinal microbiota composition in aquatic animals [21], indicating that copper not only directly affects host physiology but also indirectly regulates intestinal function through microbiota-mediated processes. At the phylum level, the intestinal microbiota was dominated by Bacteroidota, Firmicutes and Proteobacteria (Fig. 4), in line with previous studies in *E sinensis* [47]. Several important functional bacteria including *Bacteroides*, *Dysgonomonas* and *Candidatus Hepatoplasma* showed obvious and different responses in different treatments. *Bacteroides,* a key genus involved in the degradation of complex polysaccharides. It may contribute to host nutrient processing by converting indigestible carbohydrates into absorbable substrates, and its enrichment under moderate copper supplementation suggests a potential microbial contribution to intestinal nutrient processing [48, 49]. Further cluster analysis revealed that most *Bacteroides*-associated ASVs exhibited expression patterns consistent with observed changes in intestinal digestive enzyme activities and nutrient absorption-related gene expression. This two-way coordination shows that dietary copper may influence intestinal nutrient processing by enhancing the digestive capacity of the host, but also by reshaping the functional complementarity of the intestinal flora. This host‑microbiota synergy may represent a key mechanism for the improvement of intestinal function observed under moderate copper supplementation. In addition to promoting beneficial functional groups, copper also inhibits some microbial members. Copper is well known for its antimicrobial properties [50], and in this study, the relative abundances of several opportunistic or potentially pathogenic genera, including *Acinetobacter* and *Afipi*a [51], and *Pandoraea* [52], were significantly reduced after copper supplementation. Their inhibition may be related to their redox activity and antimicrobial properties, which can inhibit sensitive taxa [53, 54] and reflect the selective pressure exerted by dietary copper, which is conducive to the stability of intestinal microbial communities.

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The role of Cu in shaping microbial functional potential has been widely recognized [55]. Tax4Fun2-based functional prediction suggested that dietary copper supplementation altered the potential functional profiles of the gut microbiota in a dose-dependent manner. In the Cu28 group, predicted pathways related to bacterial chemotaxis and flagellar assembly were enriched (Fig. 5), suggesting potential increases in microbial motility- and environmental sensing-related functions. These predicted shifts may be associated with microbial responses to nutrient gradients and resource availability within the intestinal environment [56, 57]. Consistent with these changes, co-occurrence network analysis showed that the Cu28 diet was associated with increased microbial network connectivity.

The Cu28 group showed the highest network complexity, characterized by increased numbers of edges and stronger connectivity, which are generally associated with more complex microbial association patterns [49]. Notably, the increase in network complexity was accompanied by a higher proportion of negative associations, which may reflect enhanced niche differentiation among microbial taxa. Previous studies have suggested that negative associations among microbial taxa may contribute to community organization and ecosystem stability [58]. This network structure may facilitate the allocation of resources within the microbial community, allowing functional taxonomic units to occupy specific niches and participate in different steps of dietary substrate degradation. Therefore, copper-induced changes in microbial association patterns may facilitate coordinated processing of complex nutrients, thereby contributing to more coordinated microbial treatment of dietary matrices and improved nutrient processing.

Consistent with previous studies, excessive dietary copper markedly changed the intestinal microbial community structure [59, 60]. In the present study, supplementation at 38 $mg \cdot kg^{-1}$ significantly affected the α- and β-diversity, indicating that the microbial composition changed significantly under high copper conditions. At the functional level, predicted pathways related to ABC transporters and quorum sensing

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were reduced under high copper conditions. Given that ABC transporters are involved in microbial substrate transport and stress responses, and quorum sensing is associated with microbial communication and coordinated community behaviors, these changes suggest potential alterations in microbial transport- and communication-related functions [61-63]. However, these results represent predicted functional profiles and should be further validated using metagenomic, metatranscriptomic, or metabolomic approaches. Co-occurrence network analysis further supported this pattern, showing reduced network connectivity under high copper conditions compared with the Cu28 group [64]. These changes may be associated with the potential toxic effects of excessive copper, as previous studies have suggested that excessive copper can induce oxidative stress and affect cellular structures, membrane integrity and enzyme systems in microorganisms. Therefore, microbial-mediated degradation of dietary substrates may be affected under excessive dietary copper supplementation. In summary, these host and microbial responses suggest that excessive dietary copper may constrain nutrient breakdown, transport-related processes, and intestinal nutrient processing.

Despite these findings, several limitations should be acknowledged. The present study relied on 16S rRNA-based analysis and functional prediction, which provide indirect insights into microbial functions but cannot fully capture actual metabolic activity. In addition, the causal relationships between host responses and gut microbiota remain unclear. Future studies integrating multi-omics approaches and targeted validation experiments are needed to better elucidate the mechanisms underlying copper-regulated nutrient processing.

5. Conclusion

In summary, dietary copper modulates intestinal digestive function, transport-related gene expression, and gut microbiota in *E. sinensis*. The Cu28 diet enhances digestive enzyme activities, promotes the

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expression of transport-related genes, and supports a functionally coordinated gut microbiota, thereby supporting intestinal nutrient processing. In contrast, excessive $CuSO_4 \cdot 5H_2O$ supplementation disrupts these processes by impairing digestive capacity, inhibiting epithelial transport, and altering microbial function and association patterns. These findings suggest that dietary copper may influence intestinal nutrient processing through coordinated host and microbiota-associated pathways. From a practical perspective, maintaining copper supplementation within a moderate range is important for supporting intestinal function while avoiding adverse effects associated with excessive dietary copper.

CRediT authorship contribution statement

Le Chang: Formal analysis, Data curation, Writing – original draft, Visualization. **Kai Zhang:** Methodology, Formal analysis, Data curation, Visualization. **Xue Guo:** Conceptualization, Validation. **Xiaoxue Shen, Yi Zhang and Zeyuan Gao:** Writing – review & editing, Methodology, Validation. **Wenbin Liu:** Methodology, Conceptualization, Validation, Writing – review & editing, Supervision. **Dingdong Zhang:** Methodology, Validation, Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Figure

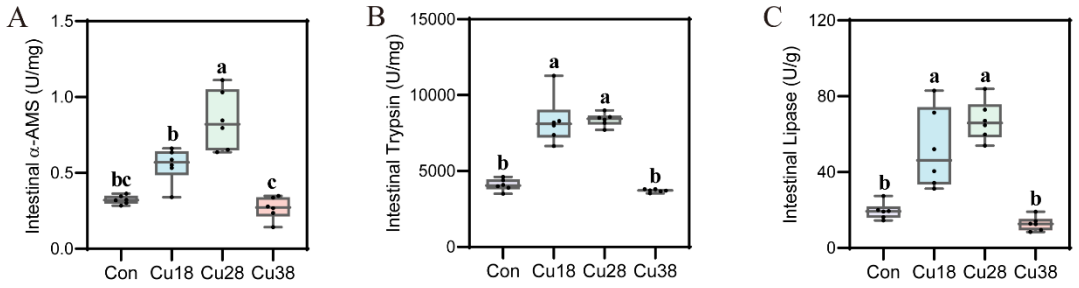


Fig. 1. Effects of dietary copper on intestinal digestive enzyme activities of *E. sinensis*. The activities of α-amylase, trypsin, and lipase in the intestinal of *E. sinensis* fed with different copper levels (Con, Cu18, Cu28, and Cu38). Different letters above the bars indicate significant differences among groups ($P < 0.05$).

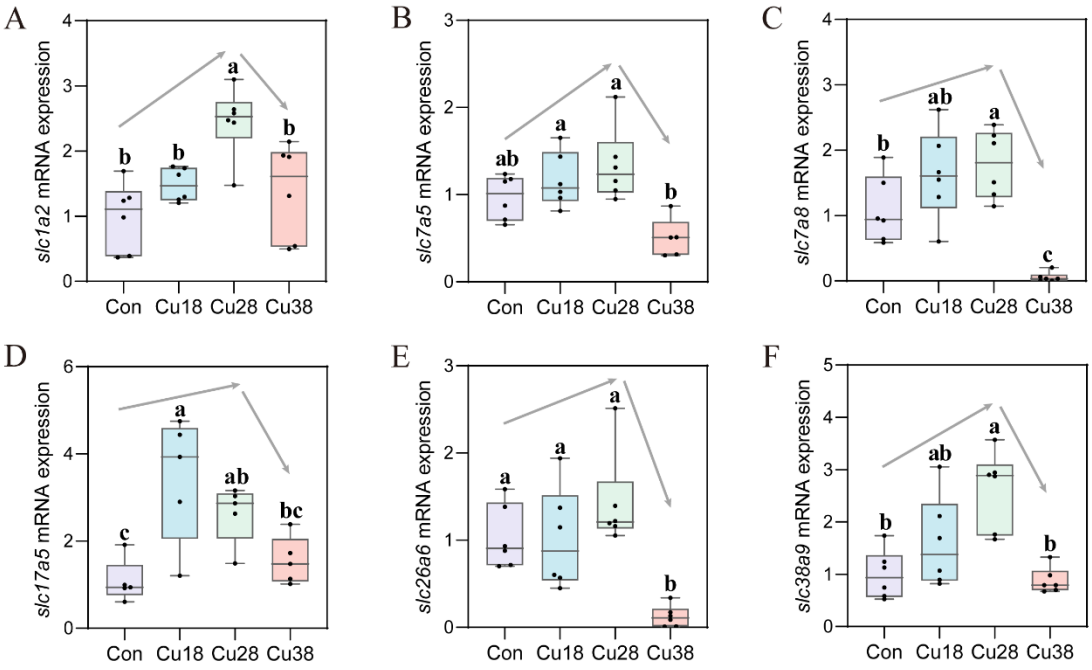


Fig. 2. Effects of dietary copper on the expression of genes related to intestinal nutrient transport in *E. sinensis*. The mRNA relative expression levels of *slc1a2* (A), *slc7a5* (B), *slc7a8* (C), *slc17a*5 (D), *slc*26a6 (E), and *slc38a9* (F) were detected in the diet with different copper levels (Con, Cu18, Cu28, and Cu38). Different letters indicate significant differences among groups ($P < 0.05$).

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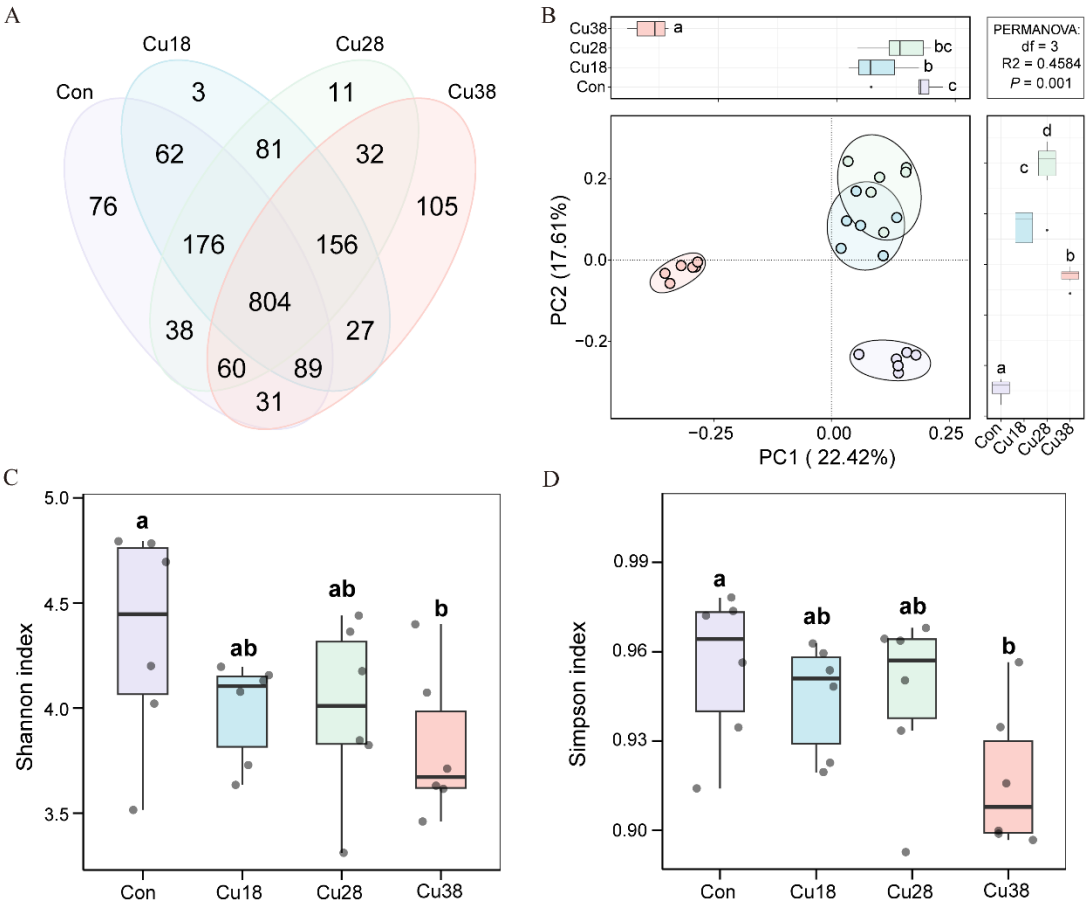


Fig. 3. Diversity and community structure of intestinal microbiota of *E. sinensis* fed diets with different copper levels. (A) Venn diagram showed the common and unique ASVs among the treatments. (B) PCoA based on Bray-Curtis distance showed β-diversity, and the difference between groups was evaluated by PERMANOVA. (C-D) α-diversity index, including Shannon (C) and Simpson (D) index. Different letters indicate significant differences ($P < 0.05$).

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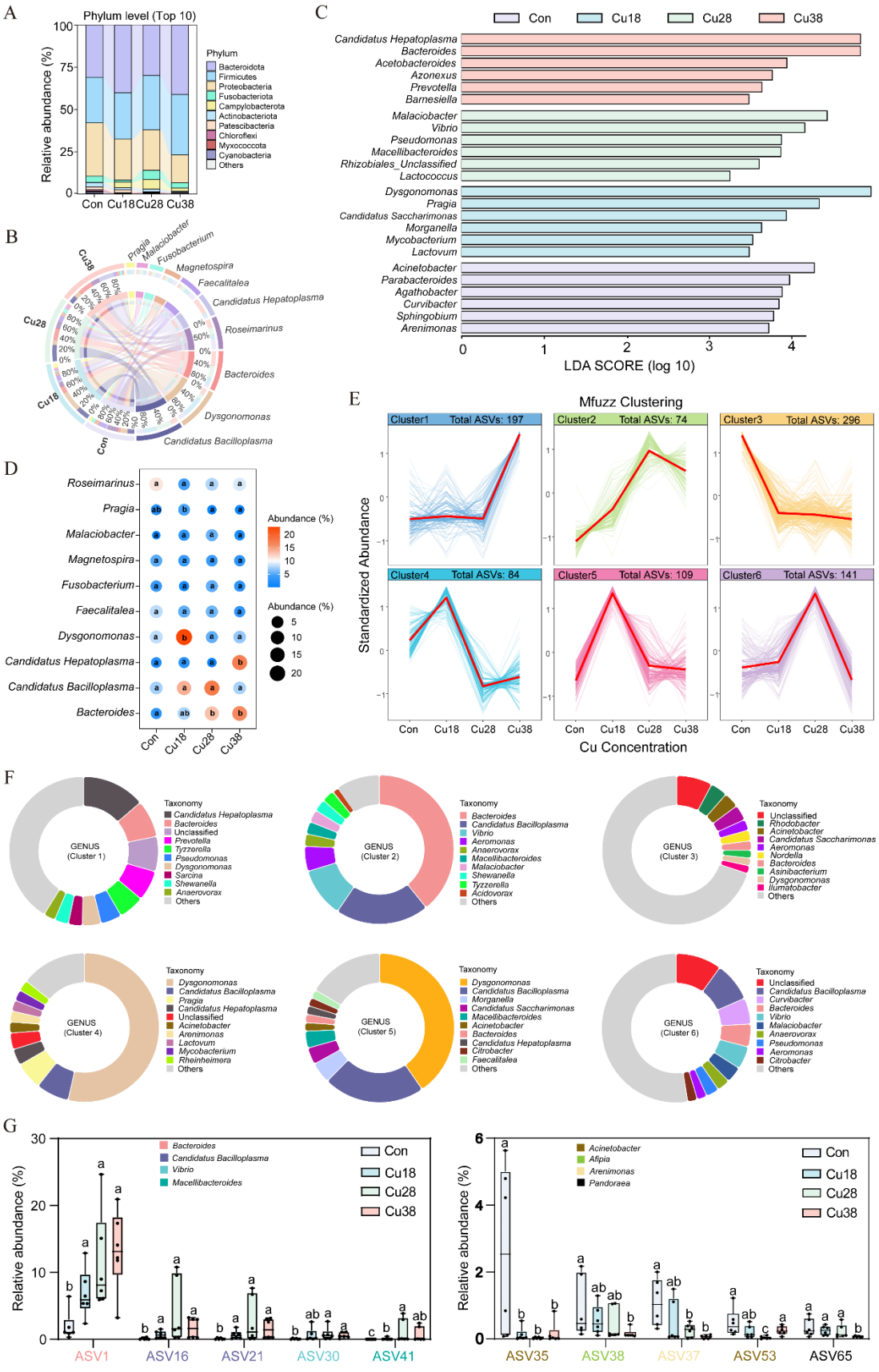


Fig. 4. Taxonomic composition and differential bacterial taxa in the intestinal microbiota of *E. sinensis* fed diets with different copper levels. (A) Relative abundance of bacterial communities at the

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phylum level (top 10). (B) Circos diagram illustrating the distribution and contribution of dominant genera among treatments. (C) LEfSe identifying significantly different taxa among groups (LDA score > 2.0), the six genera with the highest LDA scores among all significant biomarkers are displayed. (D) The relative abundances of selected genera in each treatment. (E) Mfuzz cluster analysis showing dynamic changes of ASVs with copper concentrations. (F) Taxonomic composition of representative clusters at the genus level. (G) The relative abundance of key ASVs among different groups. Different letters indicate significant differences ($P < 0.05$).

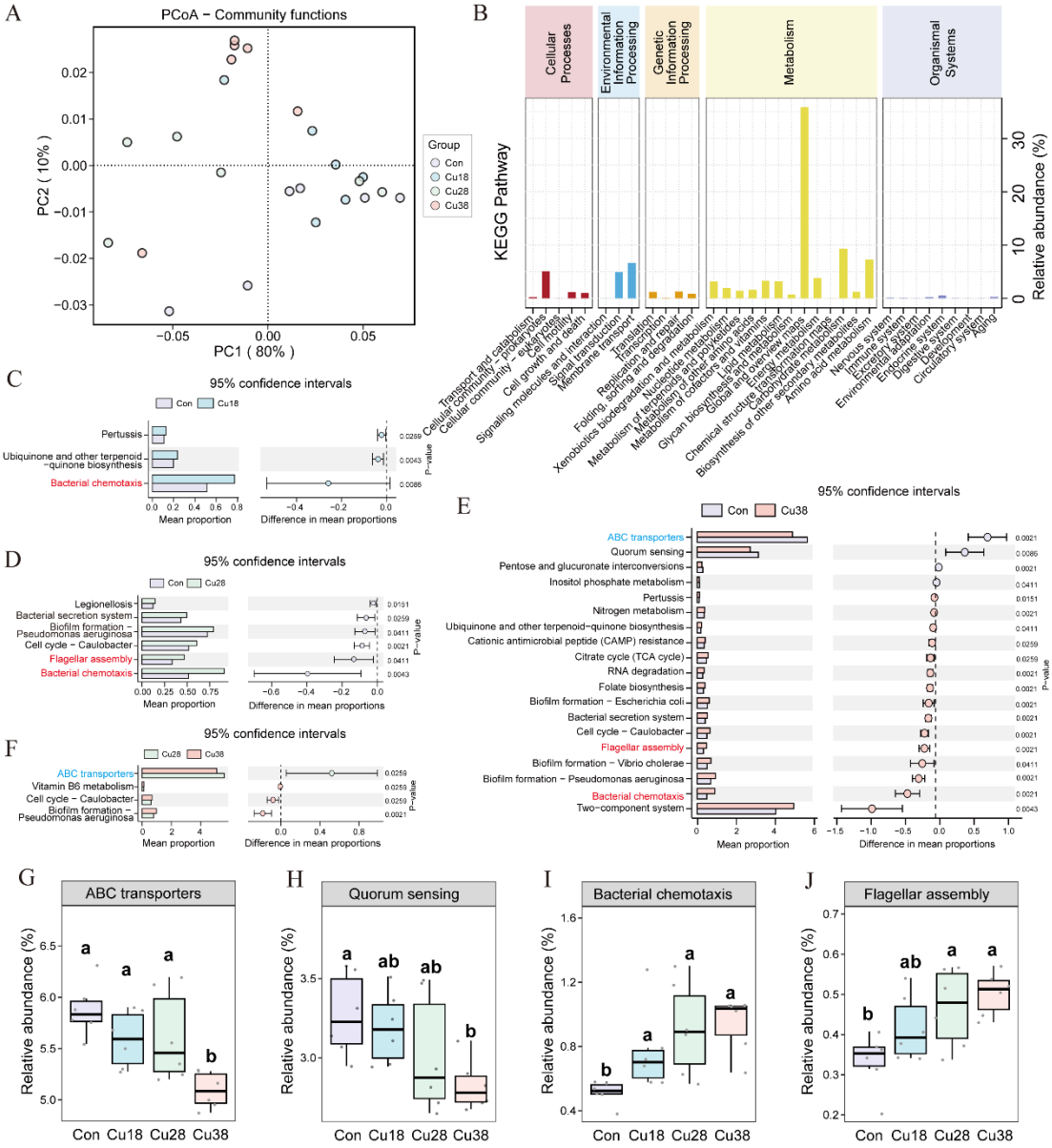
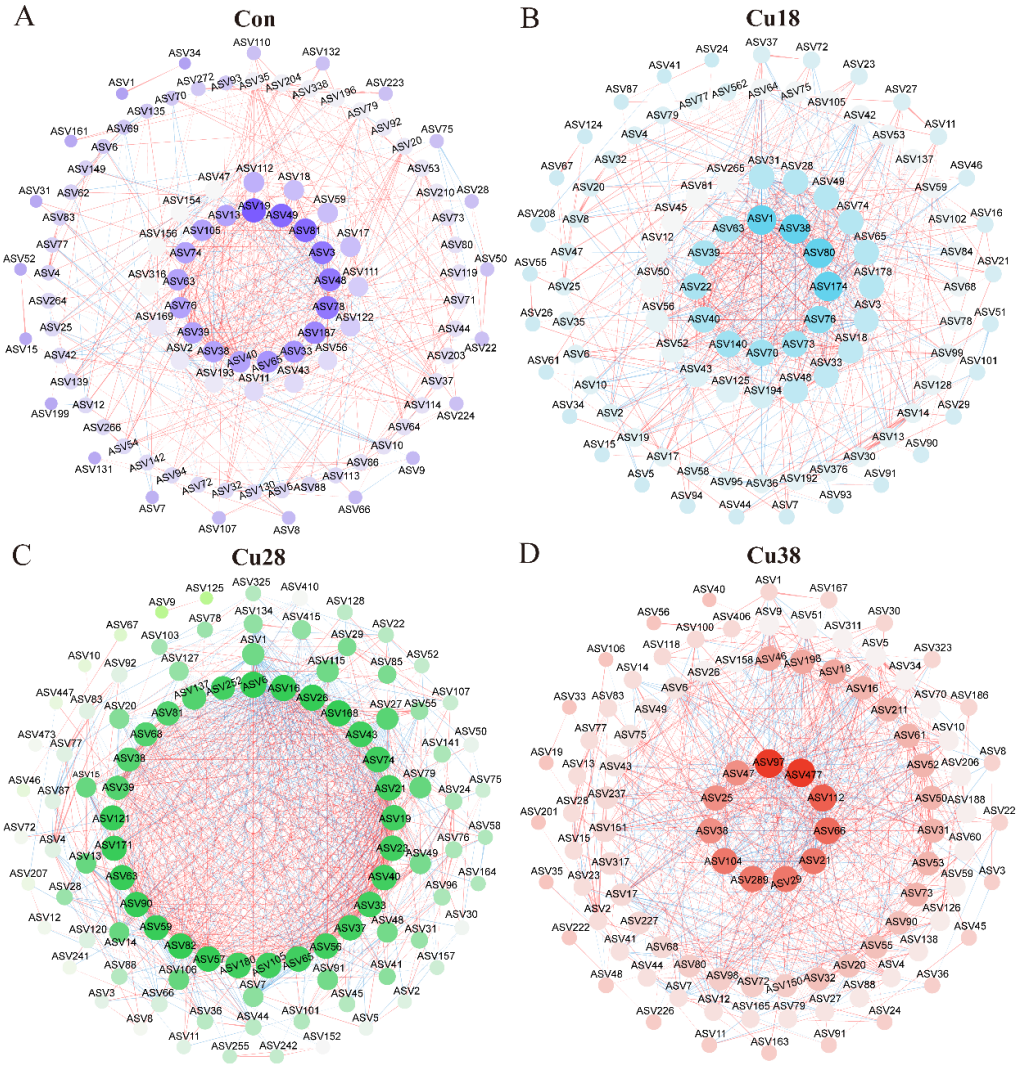


Fig. 5. Predicted the functional profiles of gut microbiota of *E. sinensis* fed diets with different copper levels. (A) PCoA for predicting microbial functions based on Bray-Curtis distances. (B) The relative abundance of KEGG pathways identified between the (C-F) groups were represented by differences in average proportions and 95 % confidence intervals. The relative abundance of (G-J) representative pathways, including ABC transporters, quorum sensing, bacterial chemotaxis, and flagellar

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assembly. Different letters indicate significant differences ($P < 0.05$).



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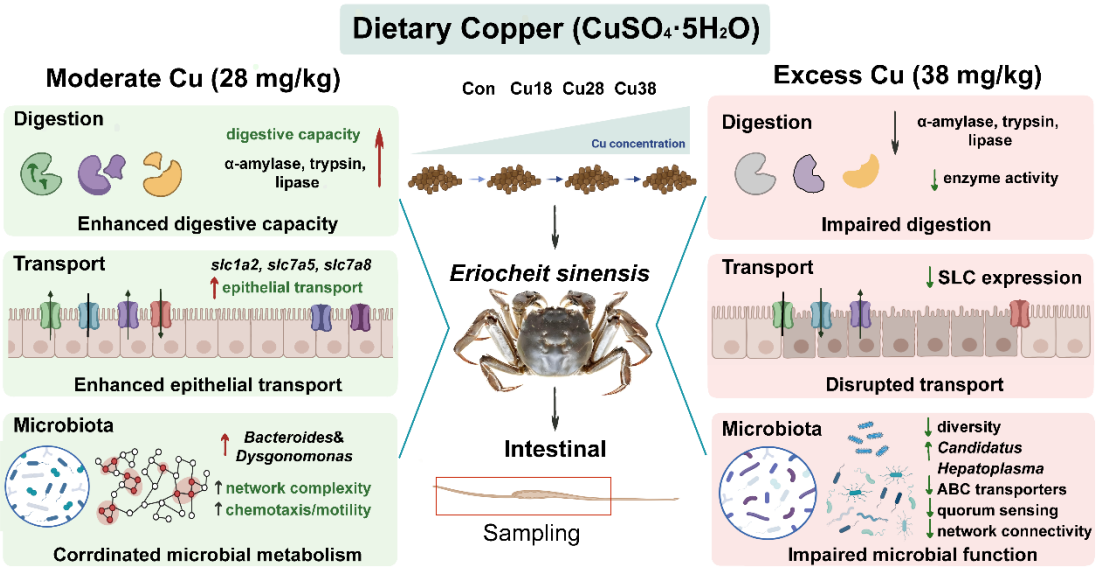
Network global properties

	Con	Cu18	Cu28	Cu38
Node	100	97	100	99
Edge	507	622	950	706
Positive(%)	75.54	62.48	63.05	64.16
Negative(%)	24.46	37.52	36.95	35.84

Fig. 6. Co-occurrence network analysis of intestinal microbiota of *E. sinensis* under different dietary copper levels. (A-D) Con, Cu18, Cu28 and Cu38 microbial interaction networks. Nodes represent ASVs, and node size represents relative abundance. Edges represent significant correlations ($|r| > 0.8$, $P < 0.05$). Red and blue lines represent positive and negative correlations, respectively. (E) The global characteristics of the network, including the number of nodes, the number of edges, and the proportions of positive and negative correlations.

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Graphical abstract



Highlights

1. Moderate copper enhances digestive enzyme activity and epithelial transport.

2. Copper reshapes gut microbiota and promotes coordinated microbial interactions.

3. Excess copper reduces microbial diversity and suppresses functional pathways.

4. Host digestion, transport, and microbiota jointly regulate nutrient processing.

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