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# Beneficial effects of AMP Scy-hepc on the gut microbiota contribute to the potential growth of *Larimichthys crocea*

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## Abstract

**Background** In light of the global ban on antibiotics in animal feed, antimicrobial peptides (AMPs) have emerged as a compelling substitute, garnering considerable interest for their potential as feed additives. Our previous study revealed that the extended (one-year) daily administration of the AMP Scy-hepc substantially boosted the growth of *Larimichthys crocea*. However, the exact influence of dietary supplementation with AMPs on the gut microbiota and the potential beneficial mechanisms remain unclear. Inspired by this, the present study endeavors to examine the alterations in gut microbiota at various gut sites in *L. crocea* following a 60-day Scy-hepc feeding regimen, building upon our prior research efforts.

**Results** Utilizing 16S rRNA sequencing, we found that dietary supplementation with Scy-hepc significantly promoted the growth of *L. crocea*, which may be caused by the remarkable changes in the microbial communities within the foregut and midgut. Notable changes were observed in *Tenericutes*, *Firmicutes*, *Proteobacteria*, *Cyanobacteria* and *Spirochaetes*. The bacterial load trends in both the foregut and midgut demonstrated a notable increase following a 60-day Scy-hepc feeding, as determined by absolute quantitative PCR analysis. Moreover, Scy-hepc supplementation increased the abundance of potential probiotics (*Rhodobiaceae* and *Planococcaceae*) and reduced the abundance of opportunistic pathogens (*Flavobacteriia* and *Mollicutes*). This led to a more intricate microbial network with enhanced metabolism-related functions, especially in lipid transport and metabolism, signal transduction mechanisms, and coenzyme transport and metabolism. And the microbial resistance (Rs) exhibits no significant change between the Scy-hepc and control groups, indicating a minimal level of toxicity to the gut microbiota of *L. crocea*.

**Conclusions** In summary, this study provides compelling evidences supporting the beneficial alteration of gut microbiota in the foregut and midgut as an underlying mechanism by which Scy-hepc feeding promotes host growth. These findings offer a novel perspective for investigating the advantageous effects of AMPs on fish health and the advancement of aquaculture.

**Keywords** AMPs, Feed additive, *Larimichthys crocea*, Gut microbiota

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## Introduction

Antibiotics, have played a critical role in feedstuffs for promoting growth and preventing diseases in various agricultural animals since the discovery of the growth-enhancing effects of Chlortetracycline in pigs in 1950 [1]. Subsequently, it is worth noting that the misuse of antibiotic can result in the emergence of multidrug-resistant bacteria and antimicrobial-resistant infections, therefore, the improper use of antibiotics remains a significant concern in industrial farming, including aquaculture [2]. For example, prolonged exposure of gut microbiota and pathogens to antibiotics can lead to the emergence and sustained proliferation of antibiotic-resistant pathogens [3]. Furthermore, the misuse of antibiotics can lead to the release of antibiotic-resistant microorganisms into aquaculture environment, drinking water, plants and aquatic products through various routes, posing a significant risk of environmental contamination and threatening human health [4, 5]. In response to the growing emergence of antibiotic-resistance in medically significant pathogens and the associated risk of transmission through the food chain to both animals and humans, in 2006, 2017 and 2019, European Union, United States and China took steps to revok their approval for the use of antibiotics in animal feeds as growth promoters [6–8]. Therefore, the primary approach to alleviate the consequences of antibiotic abuse lies in the identification of antibiotic alternatives.

Antimicrobial peptides (AMP) play a crucial role as essential components of the innate immune system, exhibiting widespread occurrence and conservation across invertebrates and vertebrates [9, 10]. Moreover, their broad-spectrum antimicrobial activity, which encompasses antibacterial activity against antibiotic-resistant strains, positions them as potential alternatives to conventional antibiotics [11]. In our previous studies, we evaluated Scy-hepc, a novel recombinant fusion peptide formed through the combination of AMP scygonadin and AMP PC-hepc, which exhibited potent activity against multiple aquatic pathogen [12, 13]. Moreover, transgenic Chlorella expressing Scy-hepc demonstrated significant protective efficacy against *Aeromonas hydrophila* infection in both black porgy (*Acanthopagrus schlegelii*) and hybrid grouper (*Epinephelus fuscoguttatus* [♀] × *Epinephelus lanceo- latus* [♂]) [14]. Additionally, Scy-hepc was the first marine biological AMP to apply for the production application security certificate in China. When administered as a dietary supplement (10 mg/kg) for *Larimichthys crocea*, it exhibited pronounced growth-promoting properties, resulting in notable activation of the GH-Jak2-STAT5- IGF1 axis, alongside the PI3K-Akt and Erk/MAPK signaling pathways [15]. However, the efficient of AMP Scy-hepc on the gut microbial community in *L. crocea* for host growth promotion remains unclear.

The gut microbiota plays a pivotal role as a potent modulator of host metabolism, inevitably impacting the health of the host through various mechanisms, including nutrient absorption and immune modulation [16]. Previous research has illustrated that feed additives like probiotics, phytochemicals, and peptides have beneficial effects on microbial community, which can enhance host growth, metabolic capacity and feed utilization [17]. For instance, the administration of probiotics (*Lactococcus garvieae*) has the potential to promote host growth in rainbow trout (*Oncorhynchus mykiss*) by significantly increasing the diversity and richness of gut microbiota [18]. Phytochemical extract (MCE), derived from plants, possesses antibacterial and anti-inflammatory properties, which has the ability to effectively treat associated diseases by modulating gut microbiota, making it extensively utilized in livestock farming [19]. 16 out of 17 bacteria from the families *Lachnospiraceae* and *Ruminococcaceae* exhibited heightened abundance in the AMP rTH2-3 supplementation group, thereby contributing to the growth of fish [20]. Thus, considering the significant potential application of AMP Scy-hepc, it is imperative to investigate its impact on gut microbiota to replace antibiotics.

In order to assess the feasibility of applying Scy-hepc in aquaculture and examine its effects on fish gut microbiota, a low dose of Scy-hepc product was incorporated as a supplement into the feed of large yellow croaker (*L. crocea*) in mariculture [15]. Here, combine our previous research and conclusions on the gut microbiota in mariculture fish and AMP feeding experiments [15, 21], we conducted a comprehensive investigation for the gut microbiota in different gut parts using 16S rRNA sequencing to investigate the impacts of Scy-hepc on gut microbiota of *L. crocea*. These valuable findings significantly enhanced our understanding of the beneficial effects on host growth from the perspective of gut microbiota. Moreover, they hold promise for advancing the application of AMPs in aquaculture and enhancing fish health.

## Results

A total of 3528 OTUs and 2,928,650 sequences were identified across all samples. On average, individuals harbored 386 ± 244 unique OTUs (Table S1). The study involved a cohort of 36 individuals, divided equally between the Scy-hepc ($n = 18$) and control ($n = 18$) groups. Within each group, 9 individuals were detected at both 1 and 60 days (Fig. 1a).

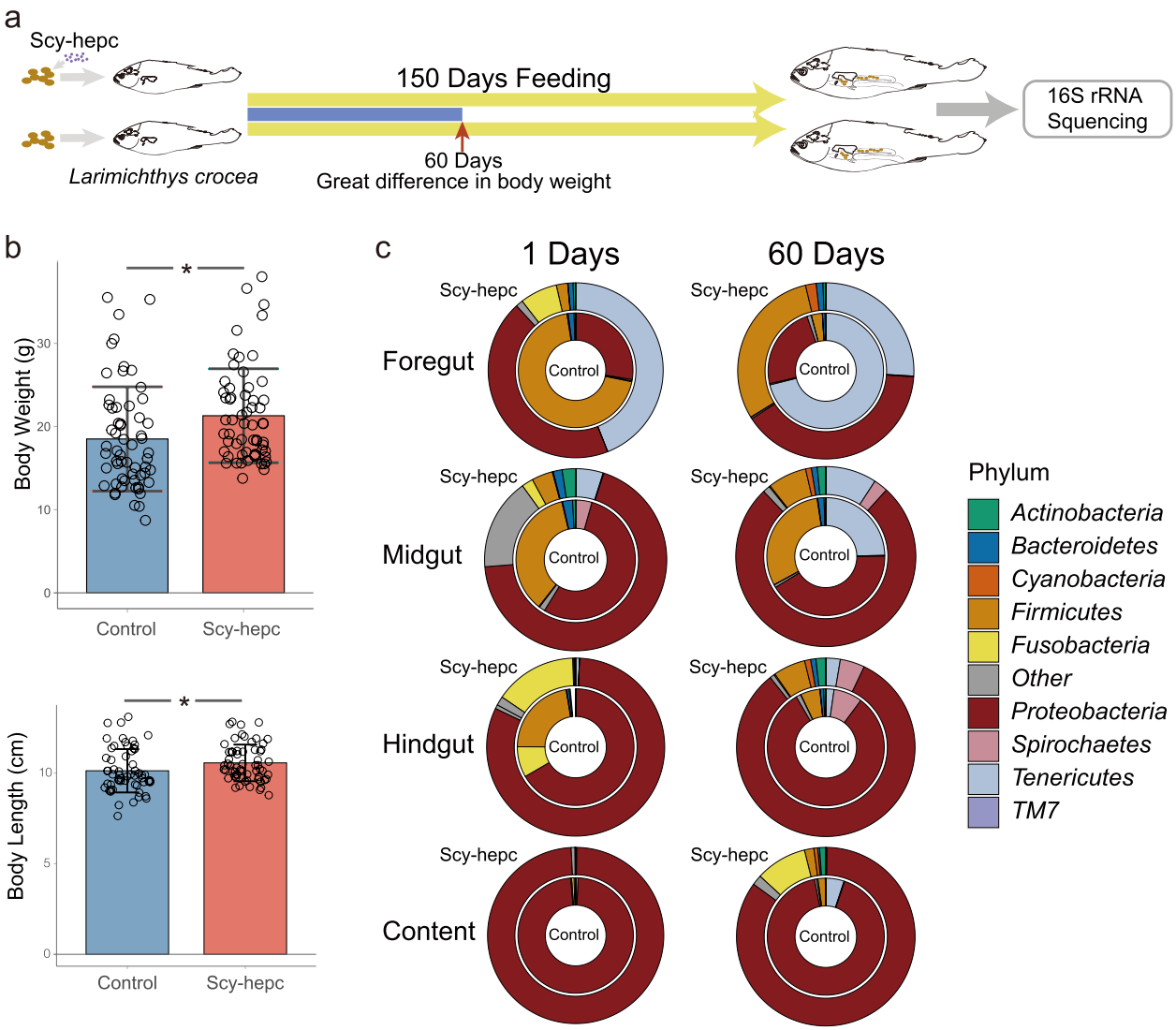


**Fig. 1** The effect of 60 days of Scy-hepc feeding on the growth performance and gut microbiota in four gut parts. **a** The experiment design flow chart in *L. crocea*. **b** Changes in body weight (BW) and body length (BL) of *L. crocea* ($n = 60$ fish per group) (*: $P < 0.05$). **c** The divergence of microbial communities among the four gut parts (foregut, midgut, hindgut, content) between Scy-hpec and control group, with the relative abundance. Only the dominant microbial phylum with top 10 of each group are plotted

### AMP Scy-hepc promote the growth of juvenile *L. crocea*

In the feeding trials conducted at FuFa Breeding Company (Ningde, Fujian Province, China), the growth performance of juvenile *L. crocea* was assessed by monitoring their body weight (BW) on days 1, 14, 30, 60, 90, 120, and 150 following the initiation of feeding (Fig. S1). After 60 days of feeding, the Scy-hepc group exhibited significantly higher growth performance compared to the control group (Fig. 1 B, $n = 60$, $P < 0.05$). The BW and body length (BL) of the fish in the control group was 18.5 ± 6.27 g and 10.13 ± 1.18 cm respectively, while the BW and BL of Scy-hepc group was 21.29 ± 5.64 g and 10.56 ± 1 cm. And our previous research also has demonstrated a notable enhancement in growth performance in *L. crocea* following 60 days of Scy-hepc supplementation [15]. Thus, gut samples of *L. crocea* were collected after feeding for 1 and 60 days to investigate the characteristics of the growth-promoting gut microbiota induced by Scy-hepc feeding.

### AMP Scy-hepc primarily alters the microbial composition of the foregut and midgut in *L. crocea*

Following the investigation of the growth-promoting effects in *L. crocea*, we expect to identify the precise locations and comprehensively examine the changes in microbial composition between Scy-hepc and control

groups. The microbial composition of the foregut, midgut, hindgut and content was assessed in *L. crocea* using the indicator species analysis (indval index) to identify gut species indicative of different gut parts (Fig. 1c, Table S2). Considering both the taxon's abundance within a community and its occurrence frequency across all communities [22].The top ten phylum, including *Tenericutes*, *Firmicutes*, *Proteobacteria*, *Cyanobacteria* and *Spirochaetes*, exhibited significant changes in the foregut and midgut and content, conversely, these core microbes displayed less variation at the phylum and family level in the hindgut (Fig. 1c and Fig. S2). Similarity percentage analysis (SIMPER) revealed that the Scy-hepc group exhibited dissimilarities 28.92%, 17.88%, 14.43% and 0.96% in the foregut microbiota, attributed to *Tenericutes*, *Proteobacteria*, *Firmicutes* and *Cyanobacteria*, respectively, compared to the control group (Table S3). The midgut exhibited dissimilarities of 17.36%, 12.42%, 10.69% and 1.17% in the microbiota, primarily attributed to *Proteobacteria*, *Firmicutes*, *Tenericutes* and *Spirochaetes.* Similarly, the content showed dissimilarities of 6.8%, 5%, 1.8%, 1.5% and 1% in the microbiota with contributions from *Proteobacteria, Tenericutes, Fusobacteria, Firmicutes, Actinobacteria.* Only 6.9%, 3.5% and 3.18% dissimilarity of microbiota in the hindgut contributed by *Proteobacteria, Firmicutes* and *Spirochaetes.* It is worth noting that, according to previous studies, for the most dominant phyla in marine fishes, AMP feeding does not change its increasing distribution trend from foregut to hindgut (Fig. S3). Notable alterations were observed in the abundance of *Acinetobacter*, *Geobacillus*, *Lawsonia*, *Photobacterium*, and *Vibrio*, at the genus level. And these alterations are distinct from the dominant microbial communities found in the aquatic environment and feed sources (Fig. S4a). *Photobacterium damselae*, *Lawsonia intracellularis*, *Geobacillus vulcani*, and *Anoxybacillus kestanbolensis* were the most significantly impacted by Scy-hepc at the species level (Fig. S4b).

### AMP Scy-hepc feeding also leads to the specific differences in taxa, diversity and bacterial loads in the foregut and midgut

The LEfSe analysis results demonstrated significantly alterations in the core microbes of the foregut and midgut due to AMP Scy-hepc treatment. This analysis aimed to identify specific taxa consistently varying in abundance between Scy-hepc and control groups, which could potentially serve as biomarkers (Fig. 2). 10 key species were overrepresented in the foregut, while the midgut exhibited overrepresented of 28 key species in both Scy-hepc and control group. In contrast, the hindgut and content only in Scy-hepc group had overrepresented of 10 and 8 key species, respectively. In the foregut, *Clostridiaceae* were enriched in the Scy-hepc group, whereas *Flavobacteriales*, *Chitinophagaceae*, *Saprospirae* and *Xanthomonadaceae* were enriched in the control group. In the midgut, *Microbacteriaceae*, *Propionibacteriaceae*, *Turicibacteraceae*, *Clostridiaceae*, *Bradyrhizobiaceae*, *Vibrionaceae* were enriched in the Scy-hepc group, whereas *Yaniellaceae*, *Coriobacteriaceae*, *Prevotellaceae*, *Bacillaceae*, *Spirochaetaceae*, *Verrucomicrobiaceae* and *Desulfovibrionaceae* were enriched in the control group.

To confirm the impact of microbial diversity divergence on the alteration in the foregut and midgut, we future measured the Beta diversity. Among the four gut parts, significantly divergence was observed in the foregut, midgut and content after 60 days of Scy-hepc feeding ($P < 0.05$) (Fig. 3a). A PCoA analysis was subsequently performed to visualize the variations in taxon composition among different gut parts following 60 days of Scy-hepc supplementation, and the differences observed in the foregut and midgut between control and Scy-hepc groups were greater than thase in the hindgut (Fig. S5).

Additionally, we quantified the total copy numbers of 16S rRNA genes using absolute quantitative PCR to assess the alteration of bacterial loads across different gut parts after 60 days Scy-hepc feeding. The bacterial load trends in the foregut, midgut and hindgut exhibited a significant increase following 60 days of Scy-hepc supplementation ($P < 0.05$), while the bacteria numbers in basal feed group remained consistent across the foregut, midgut and hindgut after 60 days of feeding (Fig. 3b). These findings further support the notion that AMP Scy-hepc feeding primarily modifies the core microbes and enhances the bacterial loads in the foregut and midgut.

### Comparison of gut microbiota composition after 60 days AMP Scy-hepc feeding

To determine the dynamic alterations in gut microbiota influenced by AMP Scy-hepc, we conducted a comparative experiment wherein all four gut segments (foregut, midgut, hindgut, and content) were sampled collectively to analyze their core microbial composition. These gut parts were considered representative of the entire gut microbiome of *L. crocea*'s [21]. These was a significant disparity in the gut microbiota composition between basal feed and Scy-hepc groups when analyzed using two different gut sampling analysis methods: analyzing all four gut fragments together and analyzing them individually (Fig. 4a). *Bacteroidetes*, *Fusobacteria* and *Other* showed high abundance in Scy-hepc group, while *Tenericutes* showed high abundance in control group. Notably, Scy-hepc had an influence on the gut microbial composition after the first day of feeding. A species classification tree was constructed to characterize the clustering

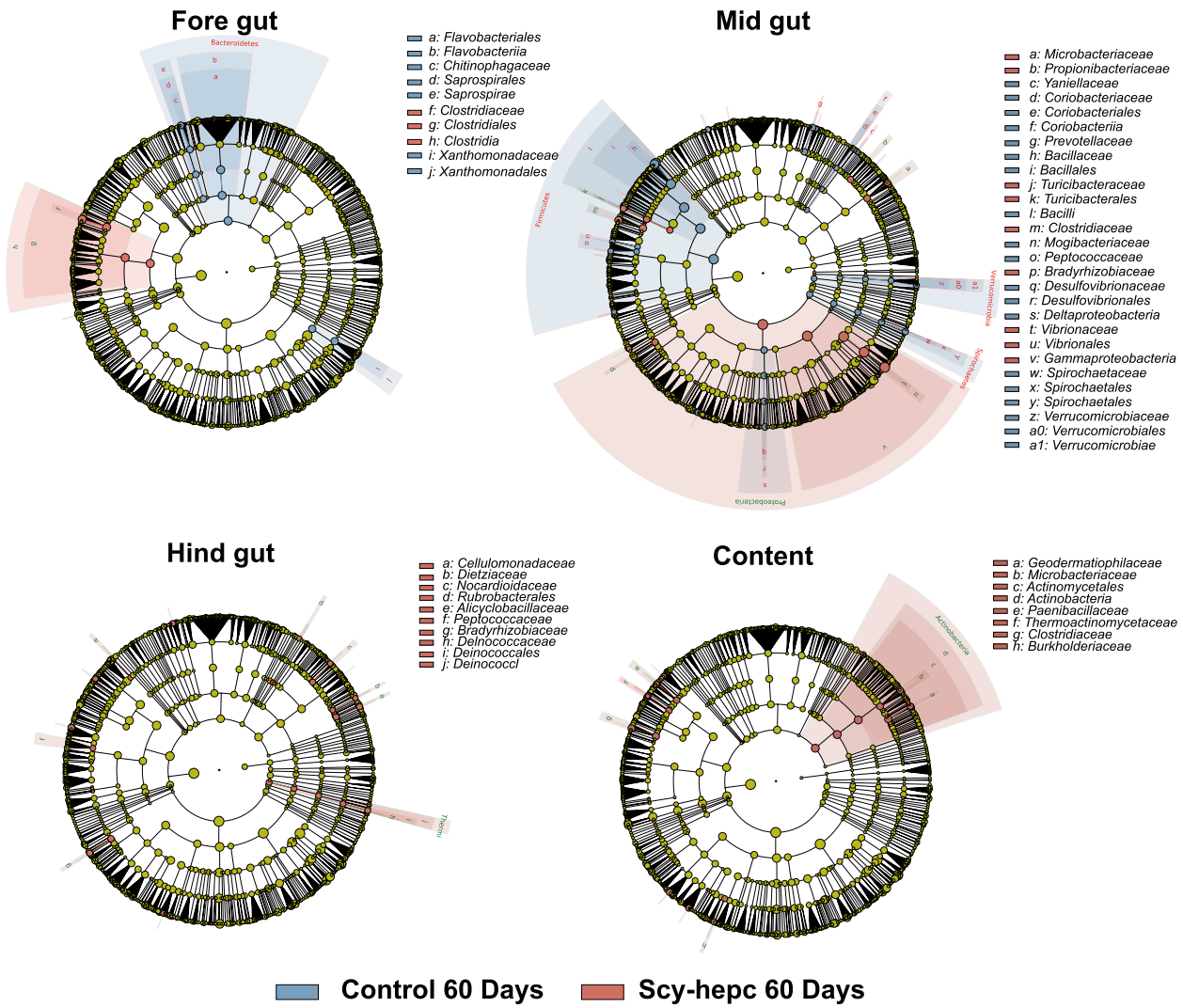


**Fig. 2** Comparison of the gut microbiota composition between Scy-hpec and control group in four gut parts. Cladogram generated from linear discriminant analysis (LDA) effect size (LEfSe) showing the relationship between taxon (the levels represent, from the inner to outer rings, phylum, class, order, family, and genus) in four gut parts respectively

and diversity of bacterial communities between 1-day and 60-day Scy-hepc feeding groups (Fig. 5). The 1-day group exhibited diverse bacterial communities dominated by various taxa, including *Pseudomonadales* (principally *Pseudomonas* and *Acinetobacter*), *Enterobacteriaceae*, *Vibrionales* (principally *Photobacterium*), *Fusobacteriia* (principally *Cetobacterium*), *Bacilli* (principally *Streptococcus* and *Bacillaceae*) and *Bacteroidetes* (principally *Chryseobacterium*). In contrast, 60-day group exhibited diverse bacterial communities dominated by various taxa, including *Pseudomonadales* (principally *Pseudomonas*, *Psychrobacter*, and *Acinetobacter*), *Vibrionales* (principally *Vibrio* and *Photobacterium*), *Alphaproteobacteria* (principally *Methylobacterium*, *Sphingomonadaceae*, *Rhodobacteraceae*, *Caulobacteraceae*), *Lactobacillales* (principally *Streptococcaceae*, *Carnobacterium*, *Aerococcaceae*). Moreover, the analysis of OTU in the gut microbial communities between Scy-hepc and control groups showed differences (Fig. S6). Specifically, the Scy-hepc group showed significantly higher number of unique OTUs (684) after 60 days of feeding. Interestingly, when comparing to the 1-day Scy-hepc feeding group, although the composition of gut microbiota changed significantly, the microbial resistance (Rs) did not show a significantly change, which suggests a

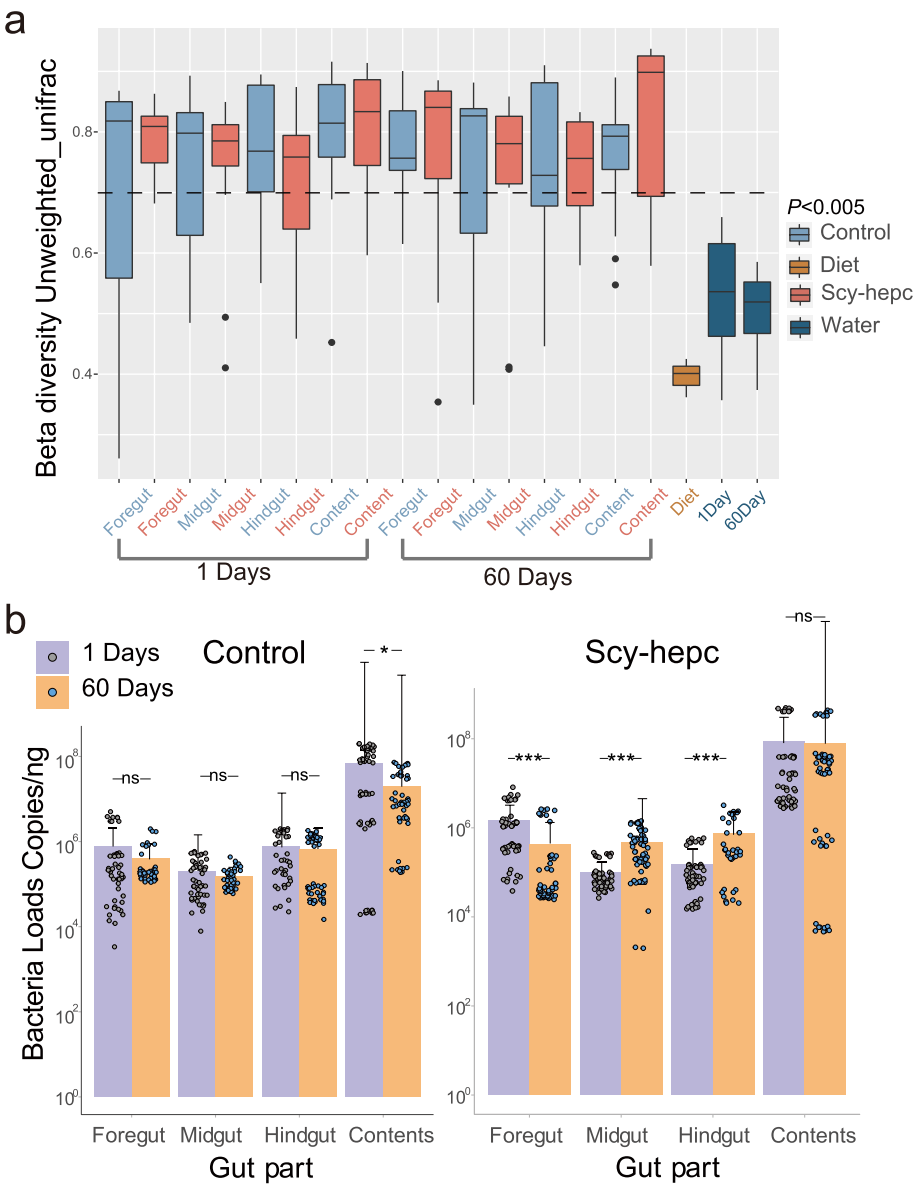


**Fig. 3** Diversity analysis and between Scy-hpec and control group in four gut parts. **a** Beta diversity Unweighted_unifrac between Scy-hpec and control group in four gut parts, differences were assessed Permanova ($n=3$). **b** Bacterial loads ($n=54$) were quantified by Q-PCR of the 16S ribosomal RNA gene. Differences were assessed by a two-tailed Mann–Whitney test (*$P \le 0.05$; ***$P \le 0.001$ and ns $P > 0.05$)

low level of toxicity to the fish gut microbiota (Fig. 4b). Similarly, there were no significant differences in microbial community resistance of the foregut, midgut, hindgut and contents (Fig. S7). Principal Coordinate Analysis (PCoA) also revealed notable distinctions between the Scy-hepc feeding and basal feed groups, including the water environment and diet (Fig. S8). And the results of CCA ordination analysis showed that $PO_4^{3-}$, $NO_3^-$ and $NO_2^-$ were the main interaction factors between the Scy-hepc group and the control group in similar ecotypes (Fig. S9 and Table S4).

**The composition of microbial communities in Scy-hepc showed more complexity than control feeding *L. crocea***

Considering the substantial impact of Scy-hepc on the gut microbial composition of *L. crocea*, we expected that differences will be observed in the gut microbial communities between Scy-hepc and control feeding *L. crocea*. With the expansion of biological datasets in terms of size and scope, scientists are increasingly adopting new techniques like network analysis to comprehend the biological complexity following diverse processes [23]. Here, we explored the bacterial co-occurrence patterns

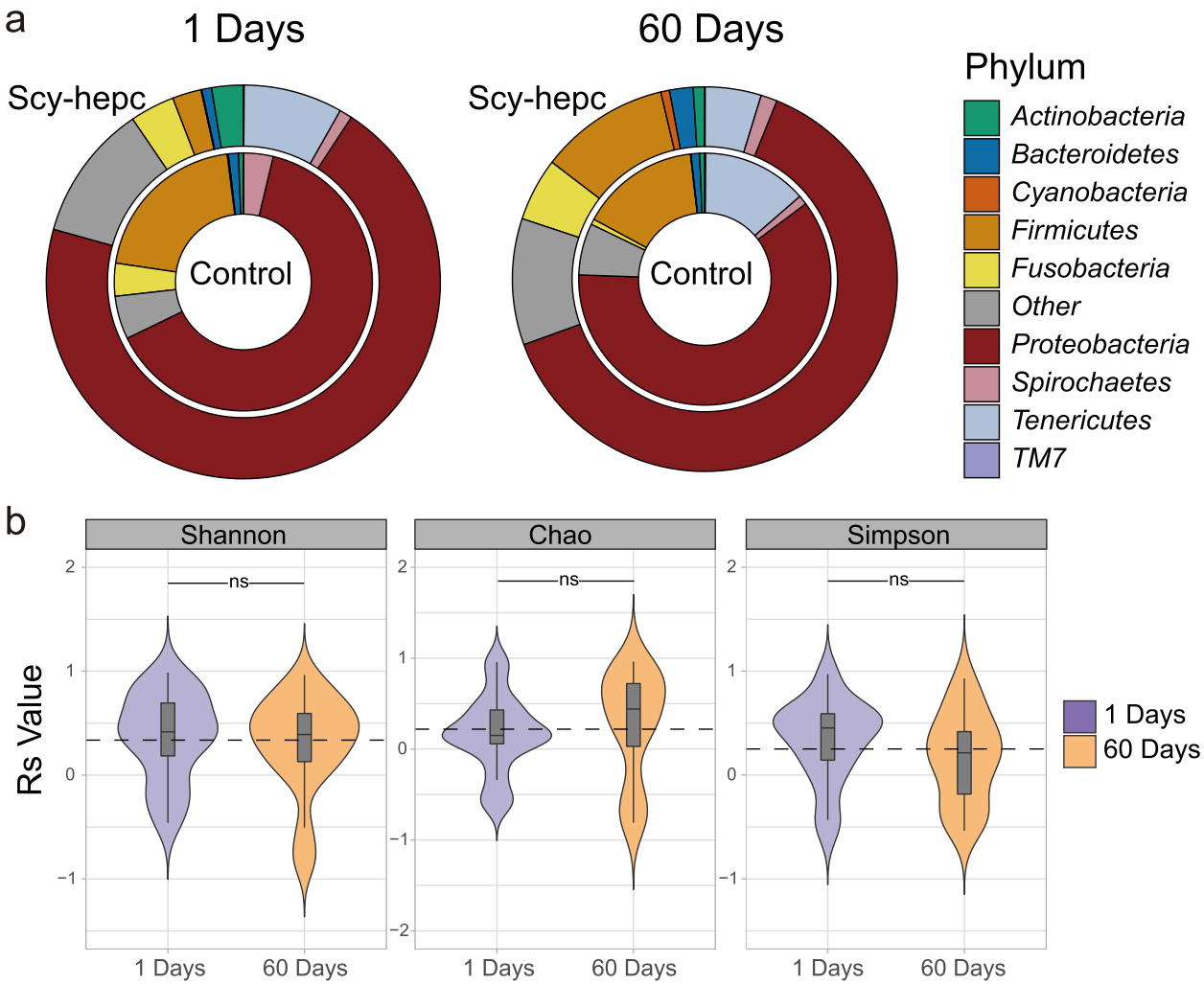


**Fig. 4** Comparison of entire gut microbiota composition after 60 days AMP Scy-hepc feeding. **a** Relative abundance with top 10 phylum of whole gut microbiota between control and Scy-hepc. **b** The microbial community resistance (Rs) between 1-day and 60-day by Shannon, Chao and Simpson index (ns $P > 0.05$)

among *L. crocea* in both the Scy-hepc group and control group through network analysis, guided by robust and statistically significant correlations [24]. The ecological networks exhibited significant divergence between Scy-hepc and basal feed groups. The network complexity of Scy-hepc group significantly increased compared to the control group, as evidenced by a higher numbers of co-occurrence events and taxa (OTU, Fig. 6). In both networks, there was a notable prevalence of positive correlations compared to negative correlations (Positive correlations > 55%, Fig. 6 and Table S5). These empirical networks also showed significant differences in average clustering coefficient (avgCC), graph density, average weighted degree, modularity and average degree (avgK). These results indicate a clear difference in the composition of bacterial community between control and Scy-hepc groups of *L. crocea.* The empirical networks exhibited a prominent "small-world" modularity and a hierarchical arrangement of their topological properties. Additional structural analysis revealed a prevalent deterministic pattern of intra-family co-occurrence within the bacterial networks. The bacterial OTUs within dominant phylums, like *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, exhibited a higher tendency to co-occur more simultaneously compared to other families. The MENs of *L.crocea* fed with Scy-hepc showed stronger species interactions and greater complexity, indicating that the strong interactions among the major species may play a crucial role in host growth and development. Furthermore, the random removal robustness analysis provided additional evidence supporting the complexity and stability of the co-occurrence network between Scy-hepc and control group (Fig. 6).

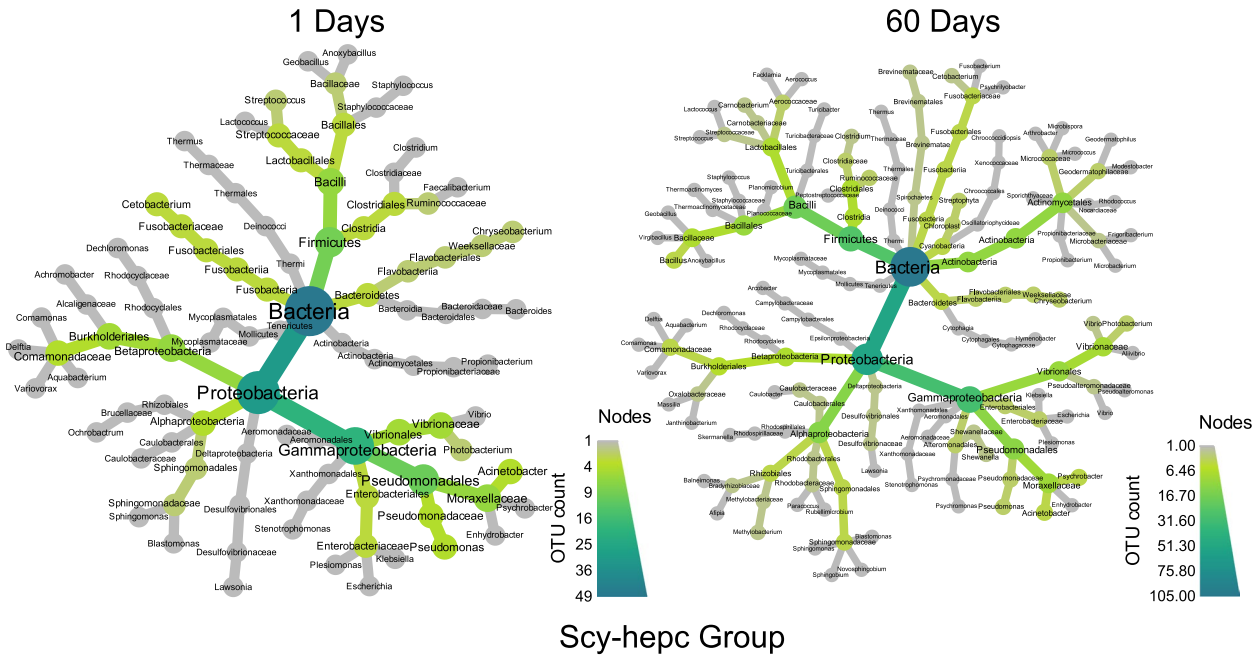


**Fig. 5** The divergence of species classification tree of entire gut microbiota between 1-day and 60-day Scy-hepc feeding. The species classification tree displayed the mean proportion of bacterial components. Nodes represent each taxonomic rank from kingdom (bacteria, center) to genus (tips of each branch). Node and edge (branch) width indicates the mean proportion of that taxon in samples belonging to that group. Size of nodes corresponds to the number of taxa and color intensity corresponds to proportions relative to bacterial samples overall. Only genus detected at ≥ 0.03% mean proportion are displayed

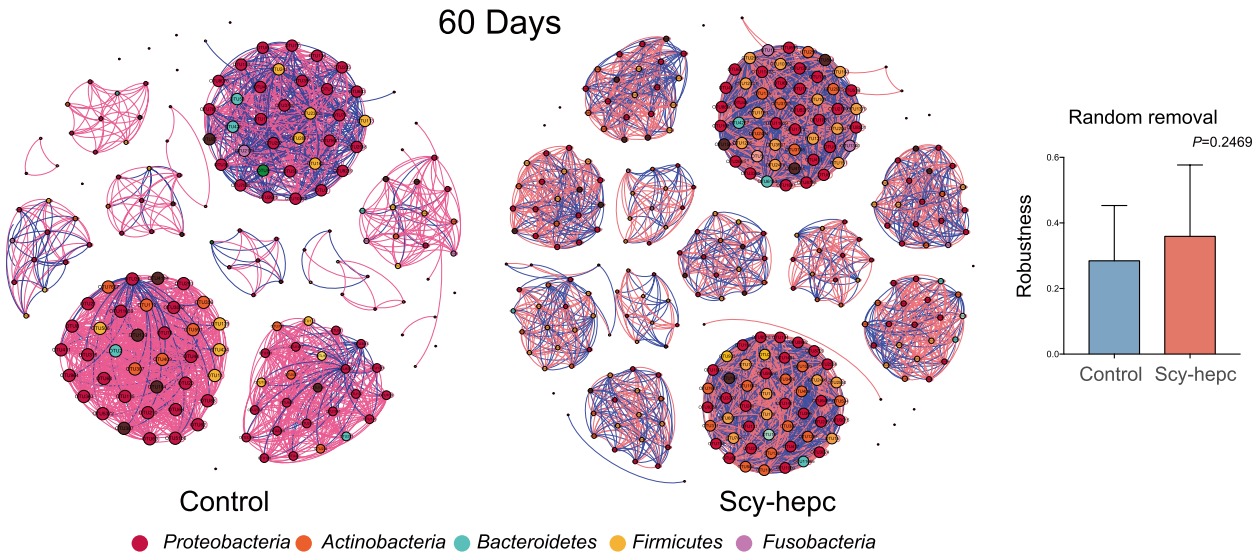


**Fig. 6** Molecular ecology networks analysis. The molecular ecology networks (MENs) of entire gut microbiota between control and Scy-hepc, which showed that the MENs of *L.crocea* fed with Scy-hepc showed stronger species interactions and more complex. And the robustness measured as the proportion of taxa remained with 50% of the taxa randomly removed from each of the co-occurrence networks

## AMP Scy-hepc primarily enhance the abundance of the potential probiotics and promote the host metabolism

To further evaluate the key species and potential functions affected by Scy-hepc feeding after 60 days, we performed Lefse, key species and Picrust analysis (Fig. 7). The results of the LEfSe analysis indicated a higher relative abundance of *Clostridiales* and *Rhodobiaceae* in

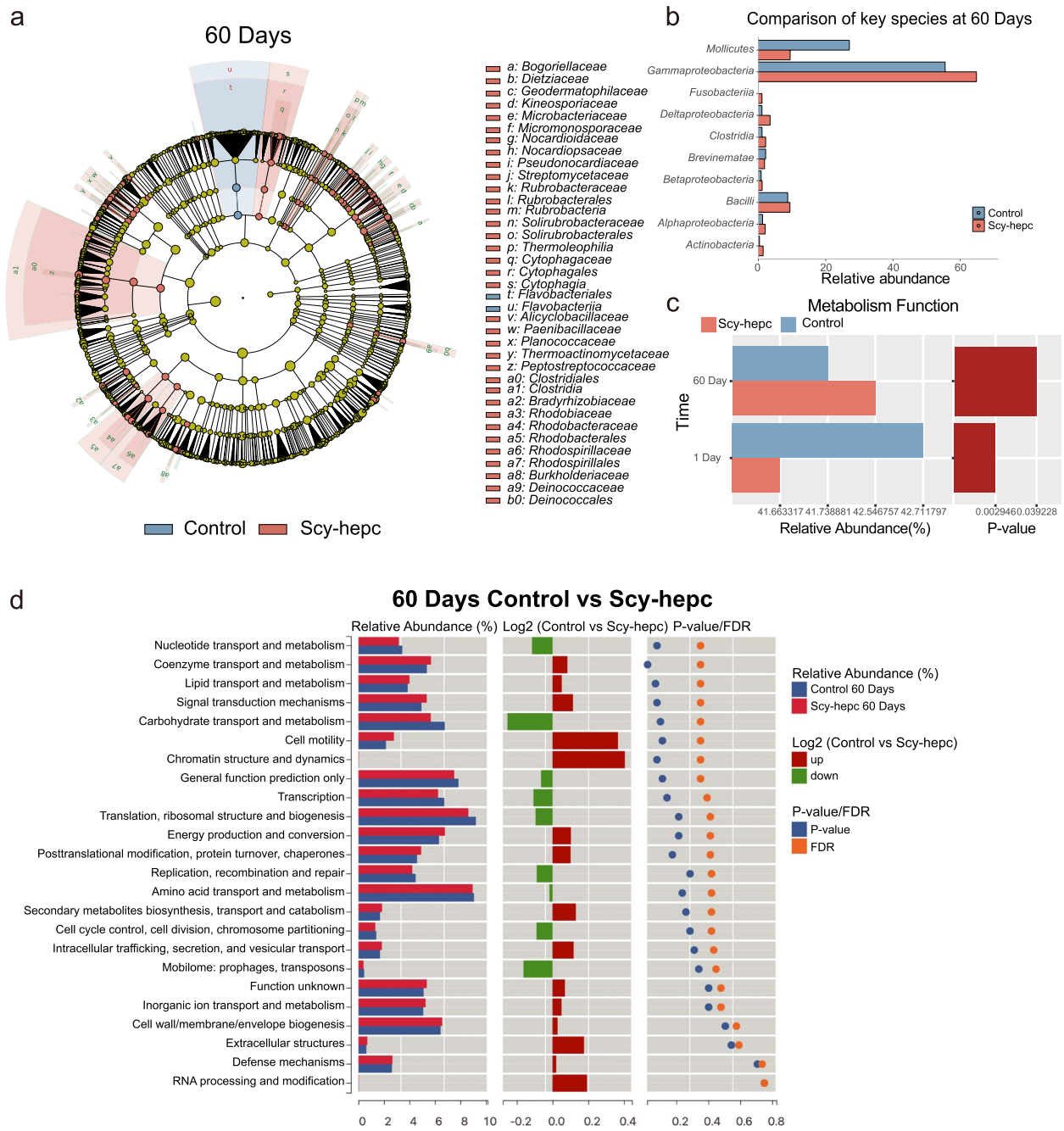


**Fig. 7** AMP Scy-hepc primarily enhance the abundance of the potential probiotics and promote the host metabolism. **a** Cladogram generated from linear discriminant analysis (LDA) effect size (LEfSe) showing the relationship between taxon (the levels represent, from the inner to outer rings, phylum, class, order, family, and genus). **b** The relative abundance of key species are strikingly different between control and SH group ($P \leq 0.05$). **c** The abundance of metabolism-related functions by PICRUSt ($P \leq 0.05$). **d** Differences in the abundance of KEGG functions at level 2 between Control and Scy-hepc group

Scy-hepc group, while the abundance of *Flavobacteriia* was enriched in control group (Fig. 7a). This suggests that AMP Scy-hepc can enhance the abundance of metabolism-promoting bacteria and reduce the potentially pathogenic species. Ten key species, including *Gammaproteobacteria*, *Fusobacteriia*, *Deltaproteobacteria*, *Bacilli*, *Alphaproteobacteria* and *Actinobacteria* were found to be overrepresented in Scy-hepc group, while *Mollicutes* was enriched in control group (Fig. 7b) Consistent with these findings, the analysis of potential

functions using Picrust indicated a promotion of metabolism functions after 60 days Scy-hepc feeding (Fig. 7c). Specifically, feeding with Scy-hepc can significantly increase the abundance of lipid transport and metabolism, coenzyme transport and metabolism, signal transduction mechanisms, cell motility, and so on (Fig. 7d and Fig. S10). Notably, changes in functional abundance varied across different gut parts, which also demonstrates the effect of Scy-hepc on the functions of microbiota in different gut parts (Fig. S11) Specifically, we observed a high abundance of lipid transport and metabolism functions in the foregut, intracellular trafficking, secretion, and defense mechanisms in the midgut, cell cycle control in the hindgut, and cytoskeletal in the content.

## Discussion

Given the escalating restrictions on antibiotic usage in aquaculture globally, there has been a growing interest in the discovery and study of antimicrobial peptides and probiotics. These promising candidates hold potential for treating pathogenic infections, promoting host immune and growth, and serving as growth additives [25, 26]. AMPs, play a crucial role in the innate immunity of animals. Consequently, they are employed as feed additives due to their broad-spectrum antimicrobial activity and minimal propensity to foster bacterial drug resistance [9]. In our prior investigation, we developed Scy-hepc, an AMP derived from marine animals, and has obtained national patents and application certificates in China, making it the first of its kind. It is produced through large-scale fermentation and utilized as feedstuff at a concentration of 10 mg/kg. Notably, Scy-hepc demonstrates the ability to enhance host growth by activating the GH-Jak2-STAT5- IGF1 axis, alongside the PI3K-Akt and Erk/MAPK signaling pathways [15]. Like many vertebrates, changes in the gut microbiota play a significant role in host health and growth [27]. Previous feeding trials have demonstrated that Scy-hepc has the ability to considerably enhance the growth of *L. crocea*. Consequently, we investigated the impacts in the gut microbiota in the presence of AMP Scy-hepc.

Previous research on the fish gut microbiota has utilized diverse gut segmentation methods and experimental approaches, however, these approaches may offer only a limited comprehension of potential functions and alterations within fish microbial communities in response to beneficial feed additives [28–30]. In our previous study, we confirmed specific distribution trends and potential functions of the fish gut microbiota across different gut parts through multidimensional investigations [21]. Building on these findings, we comprehensively examined the effects of AMP Scy-hepc on the microbial communities in the foregut, midgut, hindgut and content. The results of 16S rRNA amplicon analysis revealed a significantly divergence in the gut microbial composition and structure following 60 days of daily feeding with AMP Scy-hepc, compared to the basal feed group. Intriguingly, the gut microbiota exhibited distinct changes at the four gut sites compared to the whole gut. Specifically, there were substantial alterations in the abundance of *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Cyanobacteria* and *Spirochaetes* in the foregut and midgut, and the mainly alteration in the abundance of *Bacteroidetes*, *Fusobacteria, Tenericute* and *Other* in the whole gut (Fig. 1c and Fig. 4a). This phenomenon may be attributed to three main factors. First, differences in gut function among fish contribute to variations in gut microbiota, for example, the foregut and the midgut serve as the digestive and absorption organs in fish and possess a higher concentration of digestion-related enzymes [31]. Second, previous studies have described variations in the pre-existing indigenous core microbiota composition across different gut parts in most mariculture fish [21]. Lastly, AMP exert distinct effects on the microbial composition at different gut sites, as they display varying levels of influence on different bacteria and fungi, such as AMP scyreprocin and AS-hepc3 exhibited different antibacterial activity against various bacterial and fungus species [32, 33]. It is noteworthy that in our previous research, we observed that the antimicrobial spectrum of Scy-hepc against bacteria in vitro was not consistent with its effects on the gut microbiota in vivo. These differences may be due to the different types and numbers of bacteria targeted in the in vitro and in vivo experiments, as well as the different underlying mechanisms involved. Specifically, as an innate immune component, AMP exerts its effects in vivo through complex signaling pathways, such as NF-κB and JAK-STAT signaling [15]. Further studies are needed to explore these differences [14]. Moreover, AMP feeding can lead to an increase in bacterial load in the foregut and midgut, changing the consistent pattern observed in most mariculture fish [21]. Accordingly, more attention may be given to the selection of sampling and analysis methods in the studies examining the effects of feed additives on gut microbiota, especially in the analysis of the specific action sites of gut microbiota. Notably, some fish species, such as *Oreochromis niloticus*, possess stomach acid, which can affect growth, absorption and the composition of gut microbiota [34]. However, there is no direct evidence that *L. crocea* possess stomach acid [35]. Our additional experiments have shown that AMP Scy-hepc can be detected in the stomach, foregut, and midgut (Unpublished), which suggests that Scy-hepc can be absorbed in the intestines, despite the possible presence of stomach acid in *L. crocea*, thereby altering the gut microbiota composition. However, the extent to which

stomach acid influences AMP Scy-hepc feeding in fish requires further investigation.

Previous studies have demonstrated that feeding AMP and probiotics can alter the gut microbial composition of the host, leading to an accelerated growth rate. For example, the supplementation of probiotic HWFTM has been shown to enhance the growth rate of zebrafish by increasing the abundance of *Firmicutes* [36]. Inspired by this, the comparative experiment was conducted to more comprehensively assess the potential impact of AMP Scy-hepc on the gut microbiota of fish, specifically examining all four gut parts simultaneously. Significant divergence in microbial alterations within Scy-hepc group were observed, particularly in the phylum *Tenericutes*, *Fusobacteria*, *Proteobacteria* and *Firmicute* (Fig. 4a). This discovery corroborates findings from prior studies that have demonstrated similar alterations in gut microbiota composition within these phylums after probiotic and other AMP interventions [18, 36, 37]. Moreover, when comparing the 60-day Scy-hepc group and 60-day control group, the result of species classification tree, LEfSe and key species analysis showed a decreasing trend in the abundance of potential pathogenic bacteria and an increasing trend in the abundance of potential probiotics within the 60-day Sce-hepc group (Fig. 5, Fig. 7a,b).There are evidences that *Vibrionaceae*, *Flavobacteriia* and *Mollicutes* are opportunistic pathogens for fish, capable of causing gastrointestinal diseases [38, 39]. In contrast, *Bacilli*, *Rhodospirillaceae* and *Planococcaceae* have been applied as probiotics due to their capacity of metabolism promotion [40–42]. Consistent with these findings, an increase in the abundances of the former opportunistic pathogens and a decrease in the abundance of the latter probiotics were common features associated with Scy-hepc supplementation, which contributed to host growth.

In addition, there is growing evidence that AMP Scy-hepc enhances the stability of fish gut microbes and increases the abundance of related function potentials. A network analysis was conducted to comprehensively understand the compositions, assembly and interactions roles within the fish microbial community, shedding light on the dynamic influence between the microbial communities of 60-day control and Scy-hepc group [43]. Our findings revealed that the Scy-hepc group exhibited more complex interactions compared to control group, which fosters the stability of interaction networks and enhances the microbial community's ability to adapt to environmental changes, thus aiding in its resilience [44]. In all vertebrates, including fish, the regulation of intermediary metabolism, especially involving carbohydrates and lipids, plays a pivotal role in facilitating fish growth, as they play a vital role in maintaining the energy balance [45]. Here, we identified several potential probiotics in the Scy-hepc group that enhance metabolism (Fig. 5, Fig. 7a-b), including *Rhodospirillaceae* [46, 47]. Similarly, the results from PICRUSt prediction based on the KEEG pathway showed that Scy-hepc supplementation significantly increased the metabolism-related functions of the gut microbiota in *L. crocea* (Fig. 7c), which may contribute to the improvement in growth performance. These similar conclusions have also been confirmed in related studies on host metabolism using probiotics [48]. Interestingly, we found differences in the changes of gut microbial composition starting from day 1 of feeding, possibly influenced by the AMP's impact on the symbiotic bacteria [32]. However, the results of RS analysis indicated that daily administration of AMP Scy-hepc did not affect the resistance of intestinal microorganisms (Fig. 4b). This suggests that the impact of the AMP Scy-hepc on the gut microbiota of fish is relatively mild, unlike the addition of antibiotics or toxic substances that will significantly change the resistance of gut microorganisms [49].

As mentioned above, AMP Scy-hepc was demonstrated to promote host growth by modifying the composition of the gut microbiota. However, it exerts its positive effects on host growth by activating the GH-Jak2-STAT5- IGF1 axis, alongside the PI3K-Akt and Erk/MAPK signaling pathways [15]. Since the complex biological processes and changes involved in promoting host growth, which is the key pathway for AMP to promote host growth deserves further study.

## Conclusions

In summary, daily feeding of AMP Scy-hepc cloud play beneficial roles in the growth of *L. crocea* by altering the gut microbiota. It primarily alters the gut microbial composition in the foregut and midgut, rather than the hindgut and content. Meanwhile, the increased abundance of potential probiotics, decreased abundance of potential pathogens, enhanced microbial community stability, and improved metabolic functions may be the reasons behind the promotion of host growth through Scy-hepc feeding. To our knowledge, the effects of AMPs on microbiota at various gut sites have not been described in prior studies, which provide new insights into the study of the beneficial effects of AMPs on fish healthy and aquaculture development.

## Materials and methods

### Experimental design for feeding trials and diet preparation using AMP Scy-hepc

The AMP Scy-hepc utilized in this study was expressed in *P. pastoris,* following the procedures outlined in our previous study [14]. Specifically, the target protein

Scy-hepc was harvested from the supernatant after centrifugation using the *Pichia pastoris* secretory expression system with almost completely removal of *P. pastoris*. By optimizing the expression conditions, the majority of the total supernatant protein was Scy-hepc [14]. And the feeding trial design was detailed in our earlier study [14, 15].

In Scy-hepc group, the Scy-hepc product was acquired and b and mixed in a ratio with the commercially available basal diet to serve as feed additives, at a low dose of 10 mg/kg of feed. Control group received the same basal diet devoid of any supplementation with AMP Scy-hepc. The feeding trials on *L. crocea* utilized a commercially available formulated diet specifically designed for this species (Fuzhou Haima Feed Co. Ltd, China), which composed was as follows: crude protein ≥ 44%, crude fat ≥ 3%, lysine ≥ 1.9%, crude fiber ≤ 3%, crude ash ≤ 16%, total phosphorus 1.2–2.0%, moisture ≤ 10%. Before each feeding, the Scy-hepc additive was mixed with a suitable amount of water and subsequently adsorbed onto the granular basal diet [15].

#### Fish maintenance and Scy-hepc feeding

As our previous studies described, the feeding trials was conducted with mariculture fish *L. crocea* at FuFa Breeding Company (Ningde, Fujian Province, China). In feeding trials, a total of 40,000 juvenile *L. crocea*, each with an initial body weight of 6.3 ± 0.41 g, were randomly divided into two groups and reared in floating sea cages measuring 8 m in length, 8 m in width, and 5 m in depth. One group consisting of 20,000 fish was fed feedstuff containing Scy-hepc (at a concentration of 10 mg/kg) for a duration of 150 days, spanning from May 2018 to November 2018. The other group, comprising 20,000 fish, served as the control group and received an equivalent amount of basal diet without Scy-hepc. Initially, all fish underwent a standard 7-day temporary rearing period which were provided with basal diet. Subsequently, fish in the Scy-hepc group were givern the same amount of feedstuff containing AMP Scy-hepc (at a concentration of 10 mg/kg). All fish were fed to apparent satiation twice daily, at 07:00 and 17:00.

#### Sample collection and DNA extraction

During the feeding trials, a total of 126 fish (9 from the Scy-hepc group and 9 from the control group) and 42 water samples (collected from 0.5 m below the water surface) were regularly and randomly obtained. All fish were clinically healthy, exhibiting no signs of organ lesions. 1 L water was filtered for 16S rRNA sequencing through 0.2-mm pore polycarbonate membranes (Millipore, Massachusetts, USA). After anesthesia with ethyl 3-aminobenzoate methanesulfonate salt analytical standard (0.1 g/L, 2 min immersion, MS-222, Sigma-Aldrich, USA), the gut was carefully dissected with sterile instruments, and divided evenly into the foregut, midgut, and hindgut. Subsequently, the contents of each section were gently squeezed out and meticulously collected into sterile cryovials to ensure complete evacuation of the gut's contents, as described in our previous study [21]. Each intestinal segment from three parallel individual fish constituted an independent sample, with three samples per gut segment. A trained research technician from the institute consistently performed all treatments in a uniform manner throughout the experiment. Subsequently, the body weight (BW) and body length (BL) of fish were assessed to evaluate growth performance, and the fish guts were dissected using sterile instruments.

The microbial genome extraction for each sample followed our prior study [21], employing the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), ensuring adherence to subsequent sequencing requirements.

#### 16S rRNA real-time Q-PCR

The Q-PCR analysis was conducted using SYBR Green master mix (Thermo Fisher, USA) and primers designed specifically for the 16S rRNA sequence followed our prior study [21].

#### 16S rRNA sequencing of the gut microbiome

Based on the BW results, fish were selected at the 1-day and 60-day feeding time points, which exhibited a significant difference in BW, for 16S rRNA sequencing. PCR-amplified V4 region was selected for sequencing, utilizing the Illumina MiSeq 2000 Next Generation system. Sequencing was performed at Gene Denovo Biological Technology Co. Ltd. (Guangzhou, China). The primers, experimental conditions, procedures, and related kits employed in this study were consistent with the descriptions provided in our prior study [21], culminating in the formation of the ultimate amplicon library.

#### Sequences data processing

Data quality control (QC) and analysis were conducted using the Quantitative Insights Into Microbial Ecology (v1.8.0) pipeline [50]. Subsequently, high-quality data were integrated with tags through the FLASH software. Utilizing USEARCH (v9.0), the tags were clustered into operational taxonomic units (OTUs) with a 97% identity threshold. Representative OTU sequences were acquired and subjected to taxonomic annotations, achieved through the Greengene database (v.13.8) [51] and RDP Classifier (v2.2) software with a set confidence threshold of 0.5. To account for variations in sequence

depths across samples, all datasets were standardized by subsampling to 6,000 reads per sample. Finally, the OTU abundance for each sample and a six-level taxonomic classification spanning from phylum to species were determined.

### Comparison of gut communities and bioinformatics analysis

The qualified OTU data were utilized to compute α-diversity metrics, such as the Shannon index, using the QIIME software package [50], and the significance of differences was determined by one-way analysis of variance (ANOVA) with Bonferroni's post hoc test, utilizing SPSS software (SPSS, Chicago, IL, USA). Bray–Curtis dissimilarities were employed as β-diversity measures and subsequently subjected to principal coordinate analysis (PCoA) using the vegan packages in R and QIIME software package [50]. The indicator species analysis was constructed using the labdsv and indicspecies packages in the R software [52].

We constructed co-occurrence networks to explore the associations among microbial communities in *L. crocea* exhibiting different survival characteristics. To visualize these associations, we calculated pairwise Spearman's rank correlations and constructed a correlation matrix. A valid co-occurrence was defined as having a statistically significant correlation between species, indicated by a Spearman's correlation coefficient ($r$) > 0.6 and the $P$-value < 0.01 [53]. The nodes in the reconstructed network represented bacterial taxa (OTUs), and the edges represented highly significant correlations between nodes. To characterize the complex pattern of interrelationships among bacterial OTUs, the topological features of the networks were calculated as follows: average path length (APL), graph density, network diameter, average clustering coefficient (avgCC), average degree (avgK), and modularity (M). We conducted network analysis utilizing the igraph, vegan, and Hmisc packages within the R software [53]. Subsequently, the correlation networks were visualized through Gephi software [54], while the species classification tree was constructed using the metacoder packages in the R software [55]. The robustness of a network is defined and analyzed using the WGCNA packages in R. And canonical correspondence analysis (CCA) was conducted to determine the impact of environmental factors on the microbial composition of entire fish gut between control and Scy-hepc groups through vegan and ggplot2 packages.

All analyses were performed in R (version 3.5.1, R Development Core Team), unless otherwise specified.

## Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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**Authors' contributions**

HS and KW designed the study. HS performed the experiments, analyzed data and drafted manuscript and figures. HS, MX, HH, HP and ZA provided the sample collection. KW contributed all of reagents, materials and analysis tools. FC and KW were in charge of the funding acquisiton, supervision and correction of the manuscript. All authors read and approved the final manuscript.

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**Data Availability**

All study datas have been comprehensively incorporated within the article and/or supplementary materials/ tables. The datasets of 16S rRNA genes generated during the current study are available in the NCBI repository, accession number PRJNA1026090.

## Declarations

**Ethics approval and consent to participate**

The animal study was reviewed and approved by the Laboratory Animal Management and Ethics Committee of Xiamen University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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## Publisher's Note