Animal Microbiome

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The modulation of intestinal commensal bacteria possibly contributes to the growth and immunity promotion in *Epinephelus akaara* after feeding the antimicrobial peptide Scy-hepc

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Abstract

Background Our previous study revealed that feeding the antimicrobial peptide (AMP) product Scy-hepc significantly enhances the growth of mariculture fish through the activation of the GH-Jak2-STAT5-IGF1 axis. However, the contribution of gut microbiota to this growth enhancement remains unclear. This study aimed to elucidate the potential mechanism involved in intestinal absorption and modulation of gut microbiota in *Epinephelus akaara* following Scy-hepc feeding.

Results The results showed that a 35 day regimen of Scy-hpec markedly promoted the growth of *E. akaara* compared to groups supplemented with either florfenicol, *B. subtilis*, or a vector. The growth enhancement is likely attributed to alterations in microbiota colonization in the foregut and midgut, characterized by an increasing abundance of potential probiotics (*Rhizobiaceae* and *Lysobacter*) and a decreased abundance of opportunistic pathogens (*Psychrobacter* and *Brevundimonas*) as determined by *16S rRNA* analysis. Additionally, similar to the effect of florfenicol feeding, Scy-hepc significantly improved host survival rate by over 20% in response to a lethal dose challenge with *Edwardsiella tarda*. Further investigations demonstrated that Scy-hepc is absorbed by the fish foregut (20–40 min) and midgut (20–30 min) as confirmed by Western blot, ELISA, and Immunofluorescence. The absorption of Scy-hepc affected the swimming, swarming and surfing motility of *Vibrio harveyi* and *Bacillus thuringiensis* isolated from *E. akaara*'s gut. Moreover, Scy-hepc induced the downregulation of 40 assembly genes and the upregulation expression of 5, with the most significant divergence in gene expression between opportunistic pathogens and probiotics concentrated in their motility genes (*PomA/B, MotA/B*).

Conclusions In summary, this study shows that feeding AMP Scy-hepc can promote growth and bolster immunity in *E. akaara*. These beneficial effects are likely due to the absorption of Scy-hepc in the fish's foregut and midgut, which modulates the colonization and motility of commensal bacteria, leading to favorable changes in the composition of the foregut and midgut microbiota. Therefore, a profound understanding of the mechanisms by which

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antimicrobial peptides affect host gut microbiota will contribute to a comprehensive assessment of their advantages and potential application prospects as substitutes for antibiotics in fish health and improving aquaculture practices.

Keywords *Epinephelus akaara*, Gut microbiota, Antimicrobial peptides, Bacterial motility and colonization, Flagellar assembly

Introduction

Antimicrobial peptides (AMPs) are pivotal elements of innate immunity, playing a pivotal role in defending against pathogen invasion [1] and regulating both innate and adaptive immune responses [2]. These concise peptides exhibit broad-spectrum antimicrobial activities, offering the promising alternatives to conventional antibiotics. In recent decades, AMPs have had a significant impact on improving yields and promoting weight gain in animal husbandry[3]. For example, AMPs like LFC-LFA and rEP have been employed as feed additives for piglets [4] and *Gallus gallus* [5], enhancing animal growth and overall health. In our previous studies, AMP Scy-hepc, emerged as a promising alternative to antibiotic additives for promotion and application. Scy-hepc is a novel recombinant fusion peptide, created by combining the AMP scygonadin from *Scylla paramamosain* and AMP PC-hepc from *Larimichthys crocea*, exhibits potent activity against a range of aquatic pathogens [6]. It was the first marine biological AMP that obtained for the production application security certificate in China, and conducted large-scale demonstration experiments on various economically important marine species. It exhibited a significant growth-promoting effect when employed as a dietary supplement (10 mg/kg) for *L. crocea*, with notable activation of the GH-Jak2-STAT5-IGF1 axis, as well as the PI3K-Akt and Erk/MAPK signaling pathways [7]. Moreover, transgenic Chlorella expressing Scy-hepc showed significant protective efficacy against *Aeromonas hydrophila* infection in hybrid grouper [6]. It's worth noting that the promoting effect of growth and disease resistance of these feed additives involves not only metabolic activation but also the modulation of gut microbiota [8]. For instance, long-term supplementation with AMP rTH2-3 has shown beneficial effects on alterations of commensal microbial communities and immunomodulation in aquaculture grouper (*Epinephelus lanceolatus*) [9]. These growth-promoting additives have demonstrated their ability to inhibit sub-clinical infections, reduce growth-depressing metabolites in the gut microbiota, and limit nutrient availability for pathogens [8, 10]. However, it is still unclear if AMP Scy-hepc can efficiently prevent infection and promote growth through its influence on the colonization of host's intestinal microbiota.

Gut microbiota has arisen as a powerful regulator of both host immunity and metabolism [11–13]. However, the mechanism of AMPs affecting gut microbiota remains unclear. In the vertebrate gastrointestinal tract, the core microbial communities exhibit different habitat characteristics among individuals [14–16] and are closely linked to the host's genetics [17, 18]. These distributional partterns of microorganisms are not only essential for the microbiota but also exert inevitable effects on the host's healthy and growth [19]. For instance, different fish species show variations in the distribution of microbiota in various gut segments, reflecting the presence of distinct trends in physiologically and functionally dominant bacteria in mariculture fish [20]. Inspired by this, we formulated a hypothesis that AMP Scy-hepc may modify the colonization of commensal bacteria in different gut parts, thereby contributing to host growth and immune defenses.

Red-spotted grouper, scientifically known as *Epinephelus akaara*, holds a prominent status as an economically significant fish species. It ranks as the second-largest fish species in terms of yield and boasts high commercial value in Fujian Province, China [21]. Consequently, *E. akaara* was selected as a representative species to study the impact of feeding Scy-hepc on the gut microbiota of mariculture fish in Fujian Province. To address the hypothesis above, we conducted a series of experiments, including Scy-hepc feeding experiments, infection experiments, and absorption and action experiments, to examine the effect of altered gut microbial colonization on the host. Through these investigations, we elucidated the potential mechanism through which Scy-hepc influences the colonization of gut microbiota and subsequently promotes host health. These findings offer valuable insights to the AMPs application in aquaculture industry and improve fish health from a new perspective.

Results

Scy-hepc feeding promotes the growth performance of *E. akaara*

Here, a comprehensive investigation was conducted to examine how Scy-hepc feeding impacts the growth performance of *E. akaara*, following the workflow depicted in Fig. 1a. It is worth noting that probiotics can also serve as alternatives to antibiotic and possess the capability to enhance host growth, such as photosynthetic

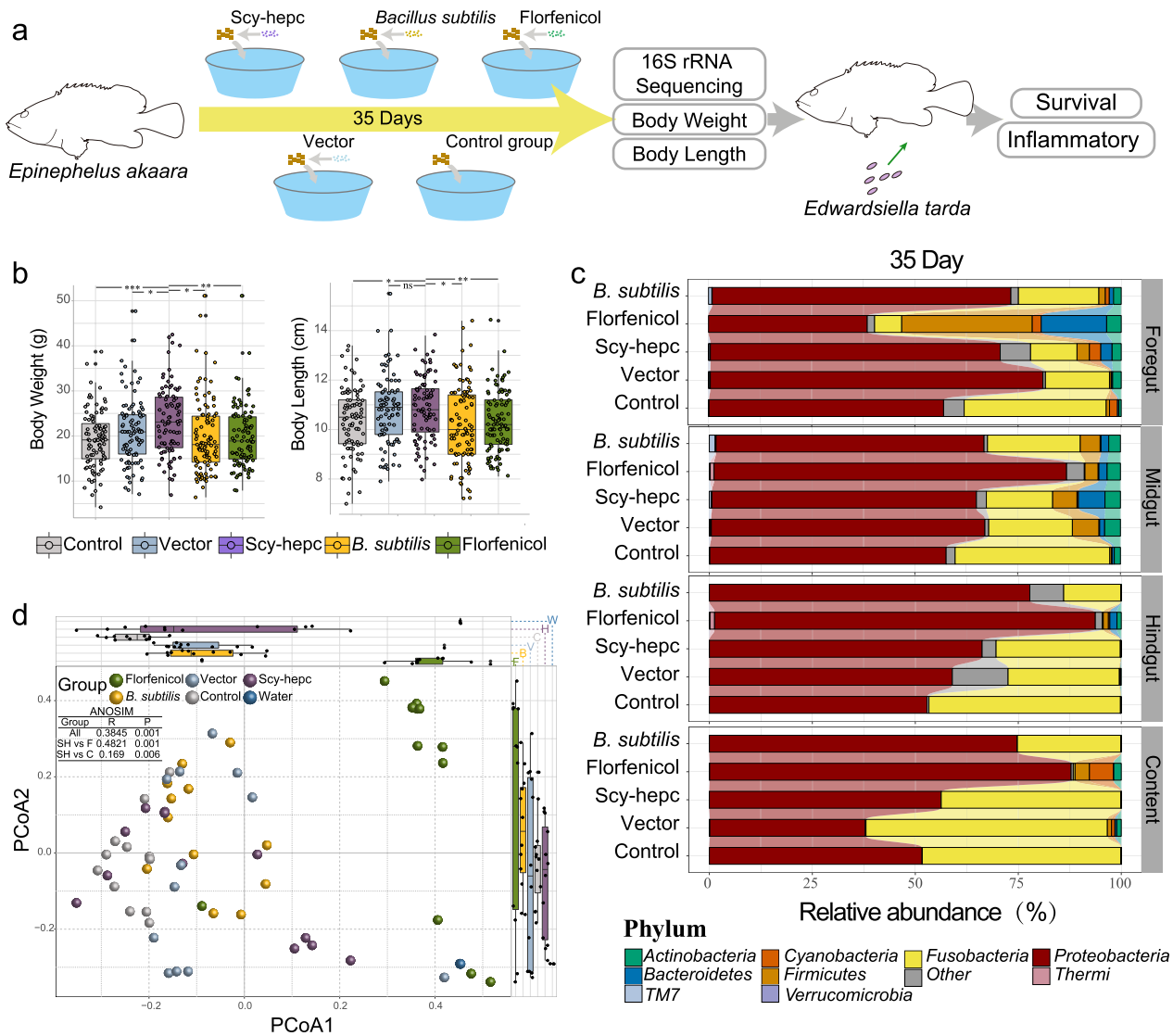

Fig. 1 Comparison of the gut microbial composition and growth performance among florfenicol, *B. subtilis* and Scy-hepc in *E. akaara*. **a**, the experiment design flow chart of different feed additives in *E. akaara*. **b**, the body weight (g) and body length (cm) of Scy-hepc, vector, florfenicol, *B. subtilis* and control group after 35 days feeding. Significant differences were indicated by asterisks: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and "ns" denoted no significant difference. **c**, the divergence of microbial communities among the four gut parts (foregut, midgut, hindgut, content) among different feed additives, with the relative abundance. Only the dominant microbial phylum with top 10 of each group are plotted. **d**, PCoA plot was constructed using OTU metrics derived from Bray–Curtis dissimilarities. Each point represents a sample. Differences were evaluated using ANOSIM, with significance set at $P < 0.05$. An R-value approaching '1' indicates dissimilarity between groups, while an R-value nearing '0' signifies a uniform distribution of high and low ranks within and between the groups

bacteria, *Bacillus* and lactic acid bacteria [22]. Inspired by this, we established distinct experimental groups to examine the effects of various feed additives on *E. akaara* (Fig. 1a). These groups included Scy-hepc, vectors (without Scy-hepc protein), probiotics (*Bacillus subtilis*), antibiotic (florfenicol) using the same concentration of action and a control group (basic diet). At the conclusion of the 35-day experimental period, Scy-hepc group exhibited significantly superior growth performance compared to *B.subtilis,* florfenicol, vector and control groups. This superiority was particularly evident in terms of weight and length gain (Fig. 1b), and the specific growth rate (39.49 ± 4.44%), feed conversion ratio (1.214 ± 0.144) further validated these conclusions (Table S1and S2).

Comparison of gut microbiota com position after 35 days of florfenicol, *B. subtilis* and Scy-hepc feeding

To investigate the dynamic changes in gut microbiota influenced by different feed additives, four gut segments (the foregut, midgut, hindgut and content) from various experimental groups were collected and analyzed using 16S rRNA sequencing [20]. The analysis yielded a total of 15, 129, 487 quality-filtered sequences across different groups, with an average of 65,511 reads. Subsequently, 20, 180 operational taxonomic units (OTUs) were clustered based on 97% sequence identity (Table S3). Among the top ten phylum, including *Fusobacteria*, *Firmicutes*, *Proteobacteria*, *Cyanobacteria* and *Bacteroidetes*, exhibited significant changes in abundance across the foregut, midgut and content. Conversely, these core microbes showed less variation in the hindgut at the phylum, family and genus levels (Fig. 1c and Fig. S1a, b). Similarity percentage analysis (SIMPER) revealed that the Scy-hepc group exhibited dissimilarities of 11.57%, 7.57%, 3.38%, 1.29%, 1.2% and 1.12% in the composition of microbiota in the foregut compared to the control group. These differences were primarily attributed to changes in *Fusobacteria*, *Proteobacteria*, *Other*, *Cyanobacteria*, *Bacteroidetes* and *Firmicutes*, respectively (Table S4). The pattens of microbial alterations in *B.subtilis* group were similar to those of Scy-hepc group, primarily affecting the foregut, midgut and contents. In contrast, the florfenicol group exhibited an opposite trend in microbial composition, characterized by significant changes in the foregut, midgut, hindgut and contents. Additionally, unsupervised hierarchical clustering analysis revealed that in the Scy-hepc and *B.subtilis* groups, samples from the foregut and midgut clustered more closely together. In contrast, in the vector and florfenicol groups, samples from the foregut, midgut, hindgut, and their contents tended to cluster within the same branch (Fig. S1c). Although there was no significant difference in alpha diversity between different groups, alpha diversity in the foregut and midgut was significantly higher in the Scy-hepc and *B.subtilis* groups than in the hindgut and contents groups (Fig. S2a, b). Compared to day 1 of feeding, significant changes were observed in the composition of the gut microbiota after 35 days of feeding (Fig. S2c, d). And the microbial composition in the *B.subtilis* group on day 35 remained similar to that of day 1. The florfenicol group exhibited the highest specificity among all treatment groups. While the Scy-hepc and *B. subtilis* groups showed higher specificity in the foregut and midgut compared to the hindgut and contents (Fig. S3a, b). Principal co-ordinates analysis (PCoA) was employed to assess variations in taxon composition among distinct feeding groups, and significant divergence was found between the Scy-hepc and the control group, as well as between the Scy-hepc and florfenicol group (Fig. 1d, Table S5). The variations across the four gut parts among the different feeding groups were also evaluated using PCoA, which showed that there was a significant separation between the foregut, midgut, hindgut, and contents of the different treatment groups ($R > 0.108$, $P < 0.05$). However, when comparing different gut parts (e.g., foregut vs midgut), the R values ranged from -0.222 to 1, with P values greater than 0.1 (Fig. S4a). Furthermore, an examination of bacterial loads in foregut, midgut, and hindgut demonstrated a significant increase in the Scy-hepc and *B. subtilis* groups after 35 days of feeding, compared to a 1 day feeding period. Conversely, there were no discernible differences in bacterial loads among the florfenicol, control and vector groups (Fig. S4b).

The results of the LEfSe analysis revealed that 11 key species were overrepresented in the Scy-hepc group, which included several potential probiotics such as *Rhizobiaceae* and *Lysobacter* (Fig. 2a). Whereas, the control group exhibited enrichment of 14 key species, the vector group had 8 key species enriched, the florfenicol group showed 26 key species enriched and *B. subtilis* group had 3 key species enriched, most of which were potential pathogens. Molecular ecological networks (MENs) were then constructed to investigate species interactions within gut microbiota after 35 days of feeding (Fig. 2b). Compared to the control group, MENs of *E. akaara* fed with Scy-hepc showed stronger species interactions and higher complexity, indicating that strong interactions among major species, such as *Proteobacteria,* might play a more significant role in maintaining host health. Notably, MENs in the Scy-hepc, florfenicol and vector groups showed stronger interactions compared to the *B. subtilis* and control groups, suggesting the complexity of gut microbial alterations and the effectiveness of feed additives on gut microbes (Table S6). Interestingly, although the composition of gut microbiota was significantly changed, the microbial resistance (Rs) did not show a significant difference, suggesting low toxicity to the fish gut microbiota (Fig. 2c).

Scy-hepc feeding promotes disease resistance capacity of *E. akaara* against *Edwardsiella* tarda infection

In order to assess the effect of different feed additives on the disease resistance of *E. akaara*, an *E. tarda* infection experiment was conducted after 35 days of feeding. The fish were challenged with a 48 h absolute lethal dose of *E. tarda* (LD100). The survival rates of the various groups 48 h after bacterial injection were 41.25% (florfenicol), 21.25% (Scy-hepc), 10% (*B. subtilis*), 12.5% (vectors), 8.75% (control), respectively (Fig. 3a). Thus, feeding *E. akaara* with Scy-hepc markedly enhanced survival rates in *E. tarda*-infected individuals, surpassing those

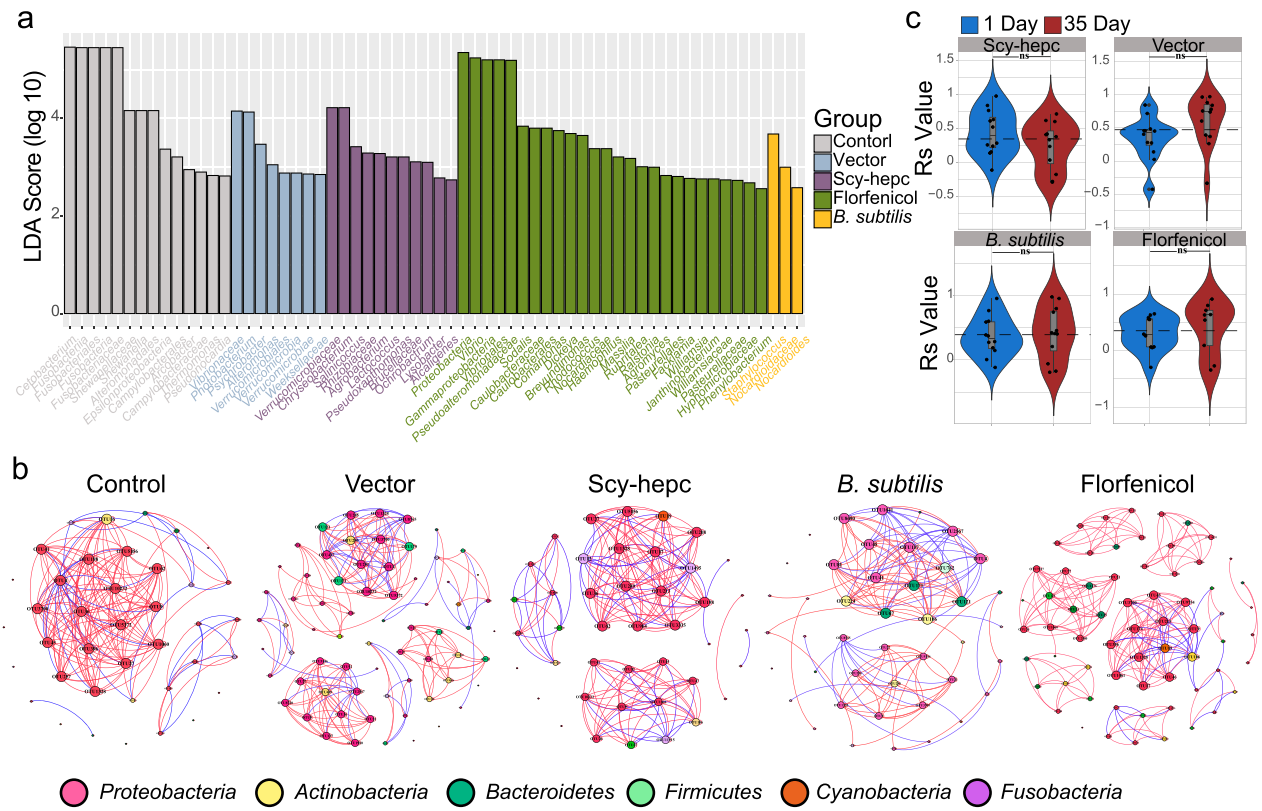

Fig. 2 Comparison of the gut microbial composition among florfenicol, *B. subtilis* and Scy-hepc after 35 days feeding. **a**, a cladogram was constructed using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) to depict the relationship between taxa. **b**, co-occurring networks of bacterial OTUs in *E. akaara* among various groups were delineated through correlation analysis. The co-occurring networks are colored by phylum. Blue edges signify negative interactions between two nodes, whereas red edges denote positive interactions. **c**, Rs comparison based on the Shannon diversity index of *E. akaara* among different feed additives group for 1 and 35 days (ns, denoted no significant difference)

observed in the *B. subtilis*, vectors, and control groups, with no significant difference compared to the florfenicol feeding group. Additionally, we examined the external morphology of the spleen, liver, and gut of *E. akaara* following infection with *E. tarda*. The results showed that intestinal congestion and swelling were more severe in the *B.subtilis*, vector, and control groups, while the Scy-hepc and florfenicol groups exhibited milder symptoms (Fig. 3b). And we subsequently conducted histological analysis of the intestine and stomach sections from different groups. These results revealed more intact tissue morphology, a lower number of infiltrated inflammatory cells and fewer goblet cells in the Scy-hepc group compared to the *B. subtilis* group, demonstrating a greater similarity of the Scy-hepc group to the uninfected control group (Fig. 3c, d).

Characterization of Scy-hepc absorption in vivo

To delve deeper into the influence of Scy-hepc feeding on the gut microbiota in the foregut, midgut and content, the absorption of Scy-hepc in vivo was characterized. The recombinant His-tagged Scy-hepc was expressed in a eukaryotic expression system and obtained through purification, following previously described methods [23]. A gavage experiment was then conducted in *E. akaara* over a 24 h period to examine the in vivo absorption of Scy-hepc. Prominent absorption signals of Scy-hepc were observed in the foregut (20–40 min), midgut (20–30 min) and hindgut (20 min) (Fig. 4a). Additionally, significant Scy-hepc absorption signals were also detected in stomach (10–30 min) and spleen (40 min-720 min) (Fig. 4b and Fig. S5a). The Western blot examination confirmed the presence of prominent absorption signals of Scy-hepc in the midgut (20 min), stomach (30 min) and serum (180 min) (Fig. 4c). Furthermore, a double antibody sandwich assay was conducted, and the results showed that Scy-hepc was absorbed in serum as a combinatorial peptide from 180 to 720 min (Fig. 4d). The results of Western blot further confirm this conclusion (Fig. S5b).

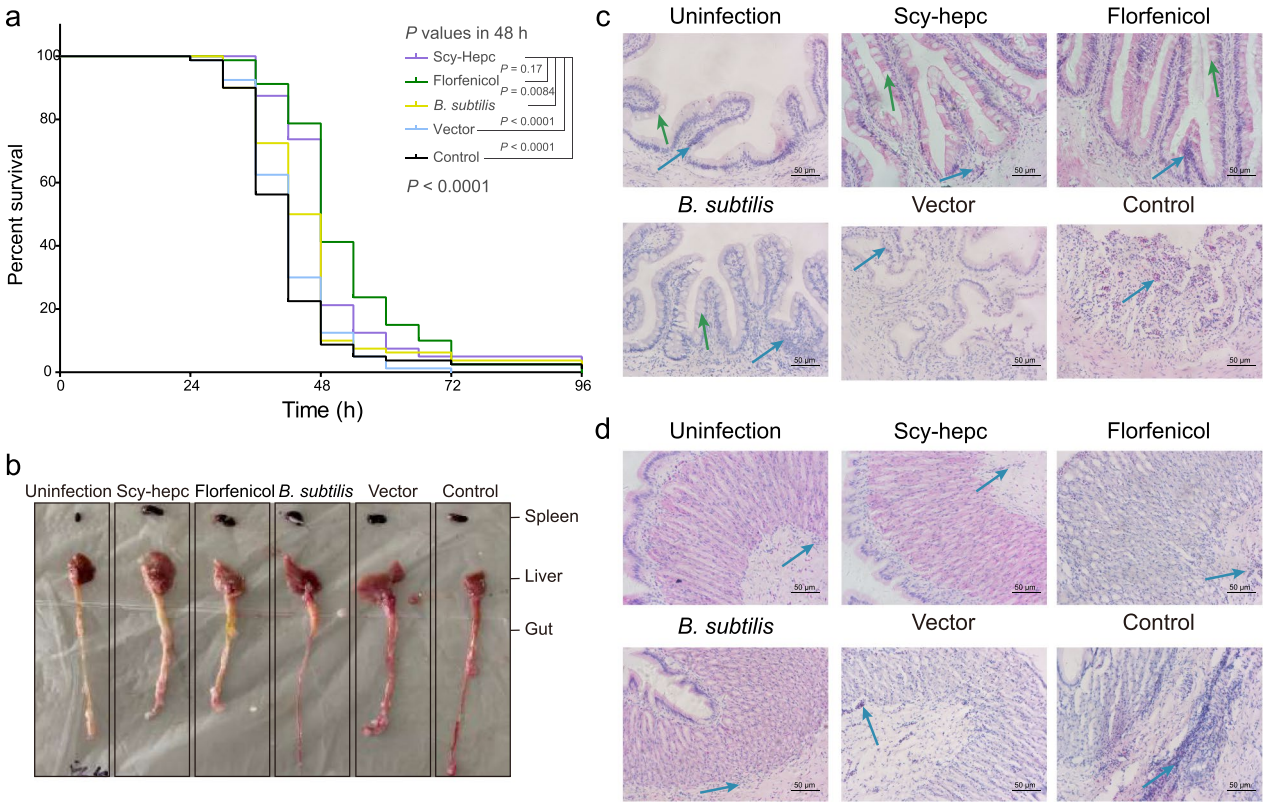

Fig. 3 Scy-hepc feeding promote the immunity performance of *E. akaara* against *E. tarda* infection. **a**, *E. akaara* of different feed additives were challenged with *E. tarda* at the 48 h absolute lethal dose after 35 days feeding. Survival curves for each experimental group were assessed through the Kaplan–Meier log-rank test. **b**, the intestinal morphology of *E. akaara* following *E. tarda*-infected. **c**, **d**, histological pathology of stomach and intestine tissues in uninfection, Scy-hepc, florfenicol, *B.subtilis*,vector and control group. The blue arrowpoint towards infiltrated inflammatory cells; the green arrowpoint towards the goblet cells

Effects of Scy-hepc on motility and flagellar assembly of commensal *bacteria*

Bacterial flagellum is a crucial organelle involved in bacterial motility and colonization, serving as a key locomotive organelle and virulence factor [24]. Given that Scy-hepc could be absorbed by the foregut and midgut, it is essential to explore whether Scy-hepc affects the motility of commensal bacteria and consequently influences their colonization. For this purpose, five commensal bacteria were isolated from the gut of *E. akaara* and cultured. The antimicrobial activity of Scy-hepc against these bacteria was evaluated (Table S7), and the results showed that Scy-hepc exhibited high minimum bactericidal concentration (MBC) and minimal inhibitory concentration (MIC) values (> 48 μM) against all five commensal bacteria. Subsequently, a Gram-negative bacteria *V. harveyi* and a Gram-positive bacteria *B. thuringiensis*, which belong to *Proteobacteria* and *Firmicutes* respectively, and are highly variable in the foregut and midgut, were selected as representative strains and sequenced by Illumina sequencing technology. The complete chromosomal genome sequencing results of *V. harveyi* and *B. thuringiensis* were presented in Fig. 5. The genome of *V. harveyi* and *B. thuringiensis* consisted of a 6,38 Mb and 5,73 Mb chromosome with 44.8% and 35.53% GC content, respectively. The chromosomes encoded 129 and 106 tRNA genes, and 37 and 42 rRNA genes, respectively. Both species exhibited polar flagella (Fig. 6a, b). We used scanning electron microscopy to examine the effects of Scy-hepc at 1 × MBC on bacterial cell morphology. The results showed that Scy-hepc reduced the flagella length in both *V. harveyi* and *B. thuringiensis* (Fig. 6c). The effects of Scy-hepc at 1 × MBC on the motility of *V. harveyi* and *B. thuringiensis* were compared. After 4–24 h of incubation on 2216E and BHI plates, significant changes in swimming, swarming and surfing motility were observed, in comparison to the control group (Fig. 6d and Fig. S6). Remarkably, Scy-hepc exhibited similar effects on bacterial motility as Polymyxin E.

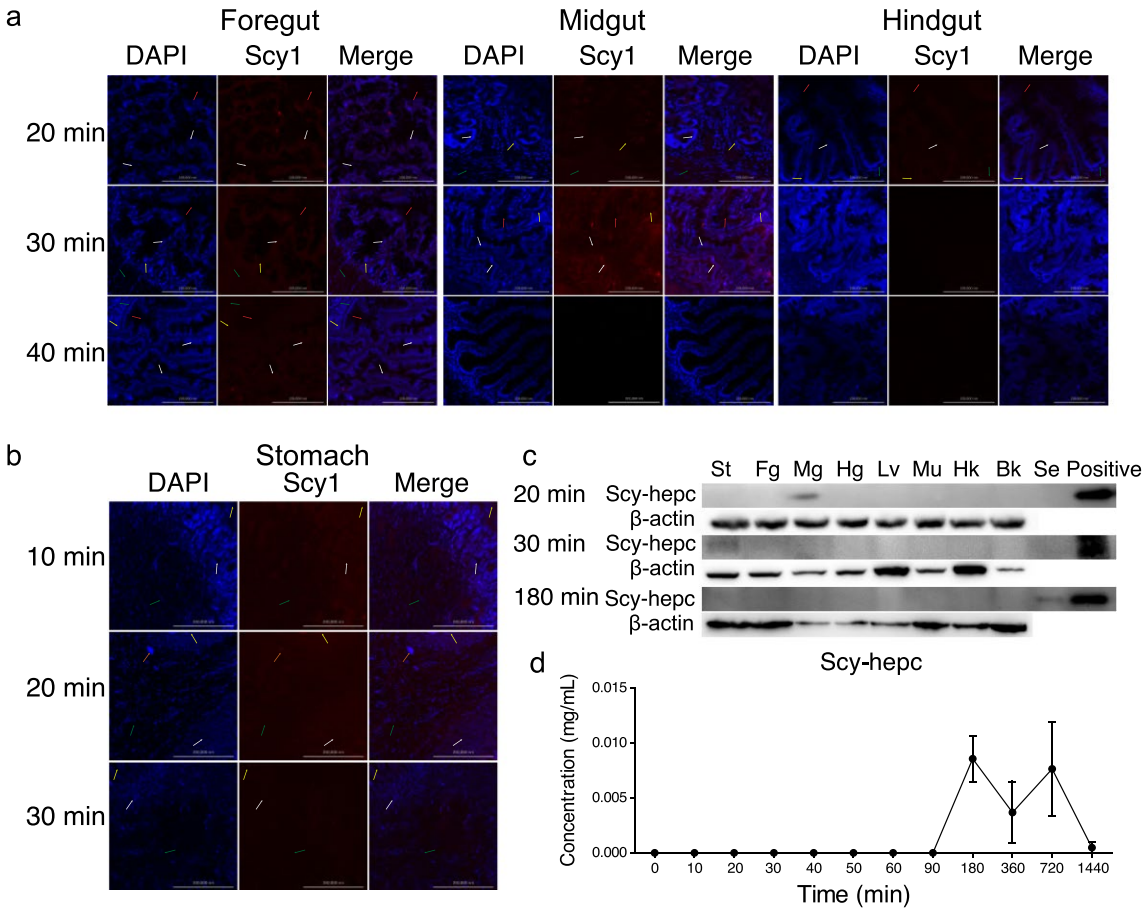

Fig. 4 Characterization of Scy-hepc absorption. **a**, representative microscopic images of the Scy-hepc absorption in different gut parts. Absorption—positive cells (red), nuclei are counterstained with DAPI (blue) (n = 6, independent animals). Scale bars: 100 μm. Light Gray arrow denote mucosa, olive arrow denote submucosa, lime green arrow denote muscularis and red arrow denote cavity. Samples were examined through confocal laser scanning microscopy. **b**, representative microscopic images of the Scy-hepc absorption in stomach. Light gray arrow denotes Gastric gland, olive arrow denotes stomach depression, lime green arrow denotes muscularis and dark orange denote blood vessel (n = 6, independent animals). **c**, Western blot showing the absorption signal of Scy-hepc in different tissues after gavage (St: stomach. Fg: foregut. Mg: midgut. Hg: hindgut. Lv: LIVER. Mu: muscle. Hk: head kidney. Bk: posterior kidney. Se: serum. Positive: positive control, n = 6, independent animals). **d**, Scy-hepc can be detected in serum through hybrid peptide by double antibody hybridization (n = 6, independent animals). Experiments replicated at least twice

Effects of Scy-hepc on flagellar assembly of *V. harveyi* and *B. thuringiensis*

Based on genomic information and motility changes in commensal bacteria, we further conducted a screening and examination of the expression of motility genes using realtime fluorescence quantitative (qPCR), Specifically, we focused on genes associated with flagellar assembly and virulence. The results demonstrated significant downregulation of twenty-three genes in Scy-hepc-treated *V. harveyi*, including genes associated with swimming motility and pathogenicity (*PomA*, *PomB*, *CdpA*, Polar flagella), flagellar biosynthesis (*FlgL*, *FlgI*, *FlgH*, *FlgG*, *FlgF*, *FlgE*, *FlgD*, *FlgB*, *FlgC*), pathogenicity (*FliD*, *FliG*, *FliH*, *FliI*, *FliM*, *FliN*, Flagella), intracellular survival and transmission (*SodB*), intestinal colonization (*ACF*), and swimming motility (Lateral flagella) ($P < 0.05$). This downregulation pattern was similar to that observed in Polymyxin E-treated *V. harveyi* (Fig. 7a and Fig. S7). In contrast, the expression of seventeen genes in Scy-hepc-treated *B. thuringiensis* showed a similar trend, including genes related to swimming motility and pathogenicity (Polar flagella), flagellar biosynthesis (*FlgK*, *FlgG*, *FlgC*, *FlgD*, *FlgF*,), pathogenicity (*FliG*, *FliM*, *FliN*, *FliE*, *FliI*, Flagella), intracellular survival and transmission (*SodB*), intestinal colonization (*ACF*), swimming motility (Flagellin) ($P < 0.05$). However, at 6 h after treatment with Scy-hepc, *MotA*, *MotB*, *FlgG*, *FlgC* and *FlgF* exhibited opposite trends (Fig. 7b and Fig. S8). These findings

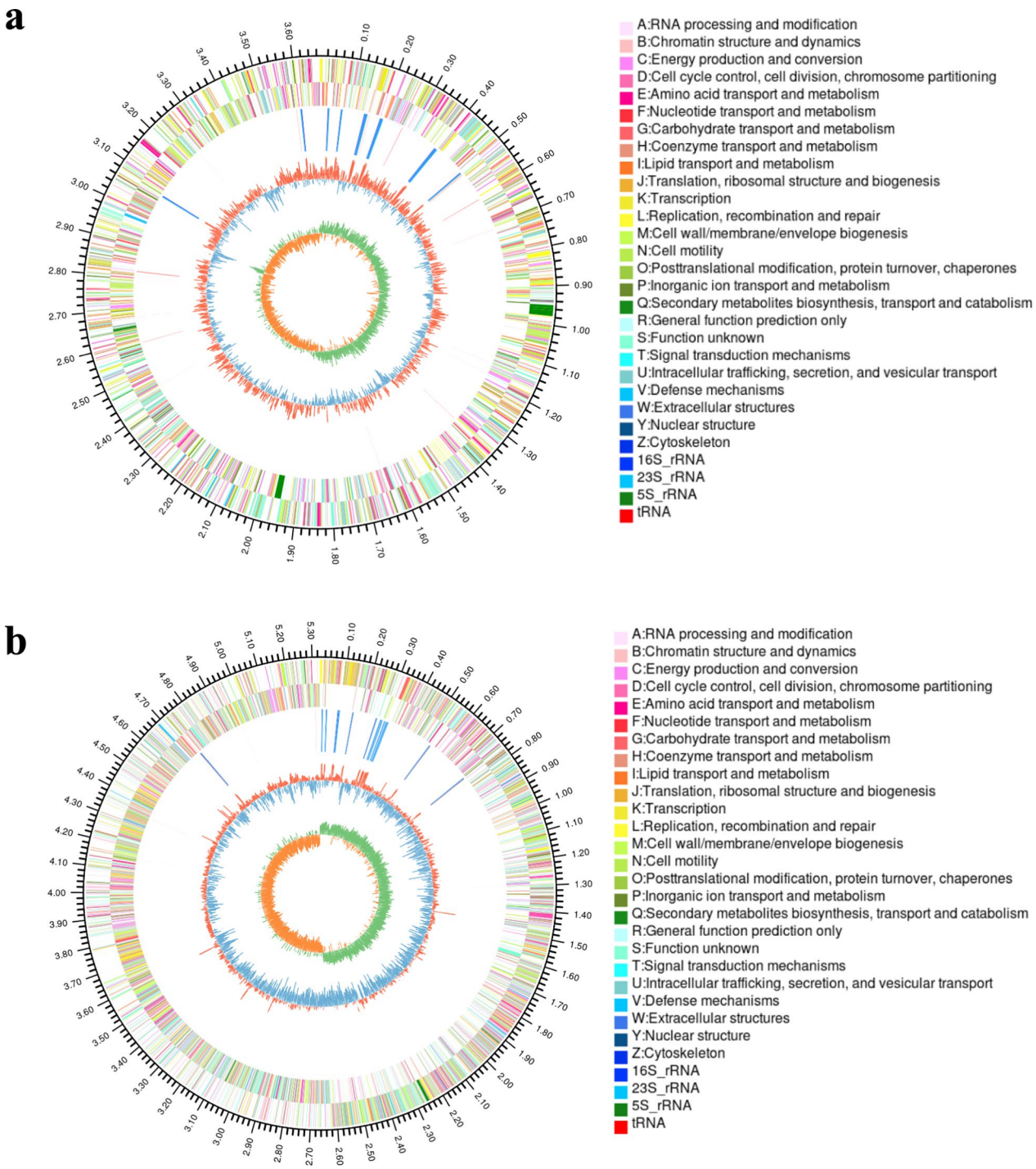

Fig. 5 Chromosomal genome of two commensal bacteria isolated from *E. akaara* gut. **a**, Circular representation of the main features of the chromosomal genome of *V. harveyi* and **b**, *B. thuringiensis* sequenced in this study

indicated that Scy-hepc may impact the motility of commensal bacteria through the regulation of flagellar assembly and virulence-related gene expression. Moreover, Scy-hepc exhibits diverse effects on flagellar assembly in different types of commensal bacteria, which may depand on the different expression patterns of motor assembly gene (*V. harveyi—PomA, PomB. B. thuringiensis—MotA, MotB*) by Scy-hepc (Fig. 8).

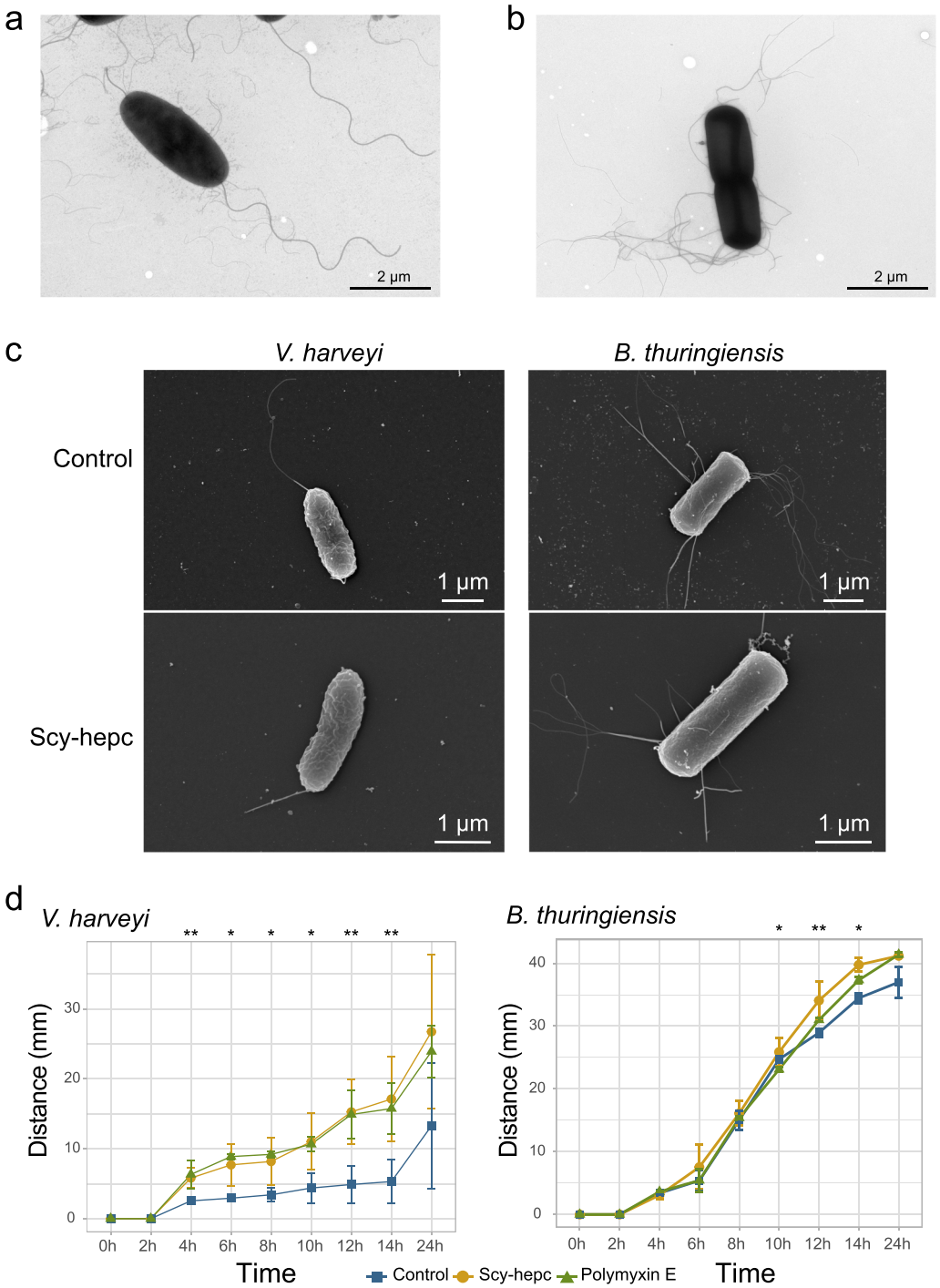

Fig. 6 Effects on the motility of commensal bacteria by Scy-hepc. **a**, Transmission electron microscope showing the morphological observation of *V. harveyi* and **b**, *B. thuringiensis* isolated from the fish gut in this study. **c**, morphological changes of bacterial cells in the presence of Scy-hepc as observed by scanning electron microscopy. **d**, mucin induced swift surface motility across diverse bacterial species. *V. harveyi* and *B. thuringiensis* were grown under surfing conditions (0.3% agar in the presence of 0.4% mucin) in 2216E and BHI with different treatment groups (n = 5, $^{*}P < 0.05$, $^{**}P < 0.01$). Experiments replicated at least twice

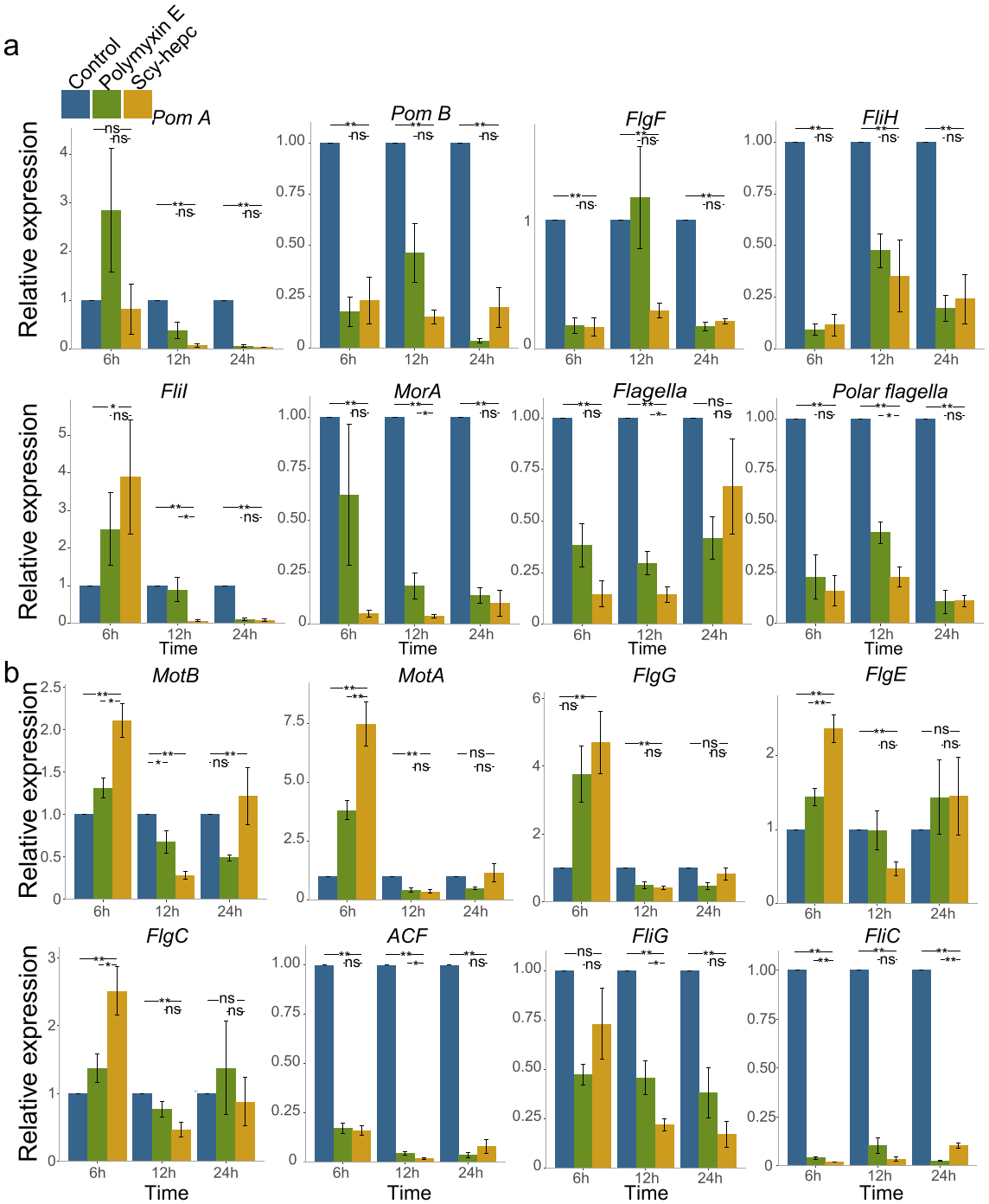

Fig. 7 Effects on the flagellar assembly of commensal bacteria by Scy-hepc. **a**, the relative expression of flagellar assembly and virulence related genes after Scy-hepc processed in *V. harveyi*. **b**, the relative expression of flagellar assembly and virulence related genes after Scy-hepc processed in *B. thuringiensis*. Differences were assessed by two-tailed Student's t test. Significant differences were indicated by asterisks: *$P < 0.05$, **$P < 0.01$ and "ns" denoted no significant difference. Experiments replicated at least twice

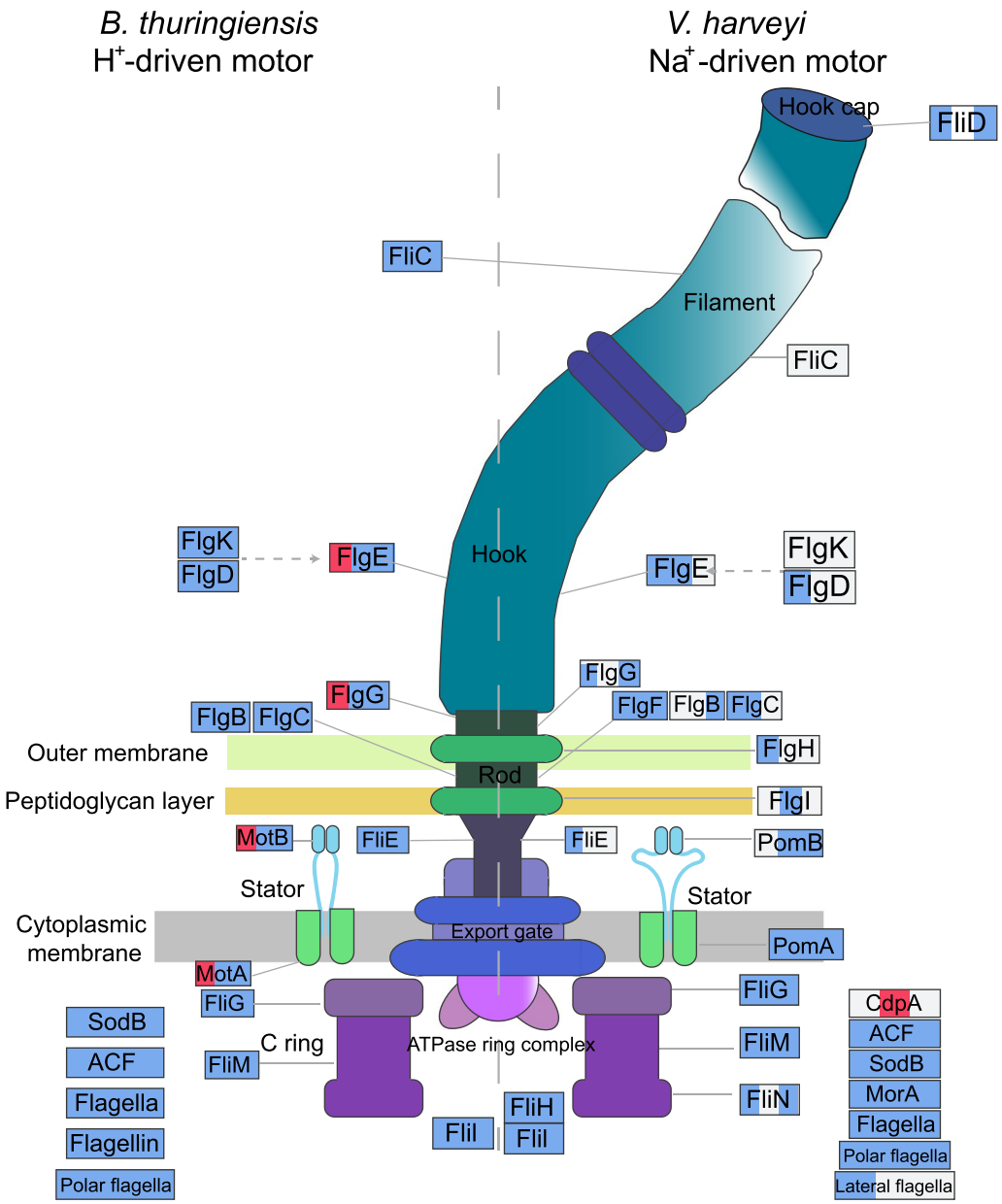

Fig. 8 Proposed mechanisms on the flagellar assembly of commensal bacteria by Scy-hepc. Gene expression patterns related to flagellar assembly by two distinct motor types. Genes highlighted in blue corresponding to down-regulated in Scy-hepc treated groups whereas red corresponded to the up-regulated

Discussion

Previous studies have pointed out that the administration of probiotics and AMPs can alter gut microbiota, which, in turn, benefits host growth and immunity [3, 22]. However, the precise underlying mechanism remains unclear. In this study, we present evidence suggesting that Scy-hepc, a promising alternative to antibiotics, can be absorbed in the fish foregut and midgut, and influence the motility of commensal bacteria motility by regulating the expression of flagellar assembly and virulence related genes. This modulation of gene expression leads to alterations in gut microbiota colonization within the foregut and midgut, ultimately promoting fish growth and enhancing immunity.

In our earlier research, many potent broad-spectrum AMPs were identified from marine animals[25–29]. Among these, Scy-hepc, one of the AMPs reported by our laboratory, had undergone extensive field trials and

practical applications [7, 30]. Recent studies have elucidated how modulating the gut microbiota through the administration of AMPs and probiotics can enhance both host growth and immunity. For example, the probiotic *Lactobacillus* has been shown to improve the defense mechanisms of *Oncorhynchus mykiss* against bacterial infection by augmenting the abundance of *Tenericutes* [31], while AMP apidaecin also showed a promoting effect on host growth and immunity in *Cyprinus carpio* [32]. In our previous application experiments, we observed a significant increase in fish body weight following Scy-hepc feeding by activating of the GH-Jak2-STAT5-IGF1 axis, as well as the PI3K-Akt and Erk/MAPK signaling pathways in *L. crocea* [7]. Given these promising results, it is imperative to delve deeper into the role of Scy-hepc in shaping the gut microbiota and its implications for host health.

In our study, we deliberately selected four distinct gut segments to comprehensively represent the gut of *E. akaara*. This approach aimed to minimize any potential biases introduced by variations in sampling methods. Significant divergence of microbial alterations in the foregut and midgut were found following Scy-hepc feeding, such as *Fusobacteria*, *Proteobacteria* and *Firmicutes* (Fig. 1c). Consistently, similar alterations in microbial composition, primarily within these phyla, were observed following the administration of probiotics and other AMPs [31–33], including the most abundant phylum in most mariculture fish species, such as *Proteobacteria* and *Firmicutes* [34, 35]. *Vibrionaceae*, *Vibrio*, *Fusobacteria*, *Psychrobacter* and *Brevundimonas* have been identified as opportunistic fish pathogens, causing gastrointestinal diseases [36–39]. In contrast, *Lysobacter*, *Rhizobiaceae*, *Alcaligenes* and *Cetobacterium* were utilized as a probiotic owing to their abilities to enhance metabolism and improve carbohydrate utilization in fish [40–43]. Consistently, the supplementation of Scy-hepc led to increased abundance of these probiotics and a decrease in the abundance of these opportunistic pathogens, which potentially contributed to enhanced fish growth. Meanwhile, an improvement in host's disease resistance, particularly in terms of resistance against *E. tarda* challenge, was observed and found to be linked to alterations in gut microbiota following Scy-hepc feeding (Fig. 3a) [44]. These findings highlight several common features, including microbial alterations in the foregut and midgut, decreased abundance of opportunistic pathogens and increased proportion of probiotics, and promotion of host growth and immunity. Moreover, the study revealed that Scy-hepc supplementation had more pronounced beneficial effects compared to probiotic and antibiotic supplementation, both in terms of growth promotion and the reduction of mortality rate during *E. tarda* infection.

The absorption of substances in the fish's digestive system varies depending on their nature. Previous studies have reported that food proteins are taken up by absorptive cells (enterocytes) in the foregut, while macromolecules are absorbed by enterocytes with high pinocytotic activity in the midgut [45]. Similarly, absorption signals of Scy-hepc were also detected in the foregut and midgut of fish that were orally administered purified Scy-hepc. This finding indicates that AMP Scy-hepc could be absorbed and potentially influence microbial alterations in the foregut and midgut. From our study, it is noteworthy that the positive signal of Scy-hepc was detected using both Western blot and ELISA with antibodies against Scy 1 and 6-His. The results demonstrated that the positive signal of Scy-hepc in the foregut was maintained for only 20–40 min, in the midgut for 20–30 min, and in serum at 180 min (Fig. 4a–d). These findings indirectly indicate that Scy-hepc has a very short in vivo maintenance time and is rapidly degraded. It is known that adaptive or specific immune responses typically develop after several days of antigen stimulation [46]. Thus, we thought that Scy-hepc probably does not have enough time to induce adaptive immunity due to its rapid degradation. However, it is worth further investigating whether specific Immunoglobulins against PC-hepc or scygonadin molecules would generate following Scy-hepc feeding.

Considering the absorption of Scy-hepc in the foregut and midgut, we further wanted to investigate the impact of Scy-hepc on commensal bacteria residing in these regions. Evidence suggested that the motility of commensal bacteria play a critical role in their colonization of specific sites, their ability to navigate to more favorable environments while avoiding unfavorable conditions, and the formation of biofilms. These processes are frequently associated with metabolic activities and the expression of virulence factors [47], and are significantly influenced by flagellar assembly and genes related to virulence [48]. Here, we observed significant alterations in flagellar assembly-related genes of intestinal commensal bacteria, namely *V. harveyi* and *B. thuringiensis,* belong to the most diverse phylum found in the foregut and midgut. The observed alterations involved genes associated with swimming motility, pathogenicity, flagellar biosynthesis proteins, intracellular survival and transmission, as well as intestinal colonization. Intriguingly, these alterations in gene expression showed two different patterns between *V. harveyi* and *B. thuringiensis,* which may apply to the effects on pathogenic and probiotics, such as Na^+-driven motor and H^+-driven motor [48]. Consistent with these findings, some AMPs have demonstrated the capability to influence the rotation of *Vibrio alginolyticus* flagella by selectively targeting *MotX*, resulting in the alteration of bacterial motility [24]. Notably, Scy-hepc

could significantly increase the motility of probiotics *B. thuringiensis* within 6 h by upregulating the expression of motor genes (*MotA* and *MotB*) and flagella-related genes (*FlgG*, *FlgE* and *FlgC*), thereby facilitating directed movement of the probiotics. In contrast, the opportunistic pathogen *V. harveyi* exhibited directional inhibition of motility after Scy-hepc co-incubation, which was attributed to the down-regulation of motor and flagella-related genes, especially the motor genes (*PomA* and *PomB*). These findings suggested that Scy-hepc has the potential to influence the motility of both probiotics and opportunistic pathogens. We hypothesize that the AMP Scy-hepc may act similarly to cyclic lipopeptide drugs, such as cyclic lipopeptides, or chemical compounds (e.g., amiloride and phenamil), by directly interacting with flagellar motor proteins (such as *MotA/B* and *PomA/B*) [24, 49, 50]. This interaction might regulate the assembly of flagella and the expression of related genes, thereby regulating bacterial motility. The mechanisms require further in-depth investigations to fully elucidate. Accordingly, AMP Scy-hepc can impact the motility of commensal bacteria, resulting in alterations to the colonization and composition of the gut microbiota.

Conclusions

In conclusion, our study sheds light on the beneficial effects of Scy-hepc on host growth and immunity in fish by inducing microbial alterations in the gut microbiota. Scy-hepc, as an antimicrobial peptide, offers a promising alternative to antibiotics. It can be absorbed by the foregut and midgut of fish and influence the motility and colonization of commensal bacteria through the regulation of bacterial flagella assembly. This resulted in a directional driving effect on probiotics and a directional inhibitory effect on opportunistic pathogens. Meanwhile, Scy-hepc could promote fish healthy by modifying the microbial composition in the foregut and midgut, increased abundance of potential probiotics, decreased abundance of potential pathogens. These findings provide valuable insights into the use of antimicrobial peptides in promoting fish health and the development of sustainable aquaculture practices. Scy-hepc's demonstrated effects offer a foundation for its future application in aquaculture and fisheries management.

Materials and methods

Study subjects and sample collection for gut microbiota analysis in feeding experiment

AMP Scy-hepc used in this study was expressed in *Pichia pastoris* based on our previous study [7]. Briefly, the target protein Scy-hepc was harvested from the supernatant after centrifugation using the *P. pastoris* secretory expression system with almost completely removal of *P. pastoris*. By optimizing the expression conditions, the majority of the total protein in the supernatant consisted of Scy-hepc [6, 7]. The vector with no inserted Scy-hepc gene was expressed and obtained using the same method as Scy-hepc.

The feeding experiments were conducted as follow: there were five feeding groups, including Scy-hepc, vector, florfenicol, *B. subtilis* and control group. To better compare the effects of different products at similar concentrations on *E. akaara*, we standardized the effective dosage to 10 mg/kg in all groups, based on the concentration of Scy-hepc in previous experiments [7]. The specific procedures for each group were as follows: in Scy-hepc group, Scy-hepc product was first obtained by *P. pastoris* expression and then mixed with the commercially available basal diet at a ratio of 10 mg/kg of feed as a feed additive [7]. In vector group, vector product was obtained and mixed with the commercially available basal diet at a ratio of 10 mg/kg of feed. In florfenicol group, florfenicol product was purchased from Hebei Jiupeng Pharmaceutical Co., Ltd, China with an active ingredient concentration of 30%, and mixed with the commercially available basal diet at a ratio of 33 mg/kg of feed, so as to reach an effective concentration of 10 mg/kg. In *B. subtilis* group, *B. subtilis* product was purchased from Henan Anmuyu Biotechnology Co., Ltd, China with an active ingredient concentration of $10^{\wedge 12}$ CFU/g, and mixed with the commercially available basal diet at a ratio of 10 mg/kg of feed. The control group received the same basal diet without any feed additives. And the diet was commercially available formulated diet for *E. akaara*, which was purchased from Fuzhou Haima Feed Co. Ltd, China. The composition of the diet was as follows: crude protein ≥ 44%, crude fat ≥ 9%, lysine ≥ 2.3%, crude ash ≤ 18%, crude fiber ≤ 6%, moisture ≤ 12%, total phosphorus ≥ 1%, calcium 0.8%-4.0%, and sodium chloride 0.3%-3.5%. Before each feeding, the different additives were mixed with a appropriate amount of water and then adsorbed on the granular basal diet.

E. akaara with an average weight of 17.19 ± 4.79 g and an average length of 9.93 ± 1.02 cm were purchased from Fufa Aquatic Products Co., Ltd. (Ningde, Fujian). *E. akaara* were acclimatized for one week in fully aerated circular seawater tanks (1200 L) at Fufa Aquatic Products Co., Ltd. Each feeding group consisted of 120 fish distributed equally in 3 tanks. During daily management, both feed and water were changed twice a day. The water temperature was maintained at 24.6–26.75 °C, salinity at 21.11–22.77‰, dissolved oxygen at 4.96–5.76 mg/L, and pH at 7.74–7.91. On both day 1 and day 35 after feeding, nine fish (three from each tank) were collected from each feeding group for 16S rRNA sequencing analysis. Additionally, 1 L of water was filtered through 0.2-mm

pore polycarbonate membranes (Millipore, Massachusetts, USA) for 16S rRNA sequencing. In this context, the fish were anesthetized, and we collected, dissected, and stored four gut sections (foregut, midgut, hindgut, and content) according to the procedures outlined in our earlier study [20]. Each research sample comprised gut sections obtained from three parallel individual fish. A trained research technician from the institute consistently performed all treatments in a uniform manner throughout the experiment.

E. tarda challenge experiments

In order to investigate the disease resistance of *E. akaara* after Scy-hepc feeding, *E. akaara* were challenged by *E. tarda* (purchased from CGMCC, no. 1.1872) after a 35-day feeding period. *E. tarda* was cultured in nutrient broth at 28 °C for 24 h, harvested by centrifugation at 4000 g and 4 °C for 10 min, washed with saline, and resuspended in saline. Healthy *E. akaara* were injected intraperitoneally with different concentration of bacterial suspensions ($10^{\wedge 4}$, $10^{\wedge 5}$, $10^{\wedge 6}$, $10^{\wedge 7}$, $10^{\wedge 8}$, $10^{\wedge 9}$ CFU/mL), and mortality was monitored and recorded daily. The absolute lethal dose (LD100) is defined as the lowest dose that results in 100% mortality of the test fish. The LD100 for *E. akaara* was determined using probit regression analysis in SPSS [51]. *E. akaara* (n = 80/group) were injected intraperitoneally with *E. tarda* at LD100 ($10^{\wedge 6}$ CFU/ fish) for 48 h, and the number of deaths and time of death were recorded, and then the survival rate was statistically analyzed.

For histological examination, approximately 2 cm fish gut and stomach segments were dissected from the control, Scy-hepc, vector, florfenicol and *B. subtilis* feeding groups (n = 3). Tissue samples were fixed in 4% paraformaldehyde (PFA) in PBS for 24 h at 4 °C. After fixation, the samples were rinsed three times with PBS and then paraffin-embedded. Tissue sections of gut and stomach were stained with hematoxylin and eosin (H & E) using previously established methods [52]. The stained sections were examined under a light microscopy (Eclipse 50i; Nikon, Tokyo, Japan).

Gut microbiome comparison

Subsequently, a comparison of gut microbiome between the different groups was performed. And the detailed procedures for DNA extraction, 16S rRNA Sequencing of the gut microbiota, Sequence data processing, Comparison of gut communities and Bioinformatics analysis, are provided in Supplementary Materials and Methods, following our previous studies [20, 53]. All analyses were performed using R (version 3.5.1, R Development Core Team), unless specified otherwise. And all the software package are provided in Supplementary Materials and Methods.

16S rRNA qRT-PCR analysis

QRT-PCR analysis was performed using SYBR Green master mix (Thermo Fisher, USA) and primers specifically designed for the *16S rRNA* gene (forward, 5′- ACT CCTACGGGAGGCAGCAGT-3′, and reverse, 5′- ATT ACCGCGGCTGCTGGC-3′), following the protocol outlined in our previous study [20].

Immunofluorescence analysis

Tissues (stomach, foregut, midgut, hindgut, spleen and liver) were collected at different time points (0 h, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 90 min, 180 min, 360 min, 720 min, 1440 min). The harvested tissues underwent fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h at 4 °C. Tissues were washed three times with PBS and transferred to 20% sucrose solution for overnight incubation. Subsequently, the tissues were embedded in OCT (Tissue Tek), subjected to freezing, and cryosectioned into 10 μm slices. Frozen tissue sections underwent fixation in a 4% paraformaldehyde solution for 15 min, followed by PBS washing, incubation with a 0.2% Triton solution, and pre-incubation with 2% bovine serum albumin for 1 h to prevent nonspecific staining. Subsequently, sections were incubated overnight at 4 °C with antibodies targeting Scy1. The polyclonal antibody against SCY1 (dilution 1:500) was generated using a previously established method [25]. Following three washes with PBS, the sections were incubated in darkness for 1 h with Alexa Fluor 594-conjugated IgG fluorescent secondary antibodies (dilution 1:5000, A11058, Invitrogen). Following three additional PBS washes, the sections underwent counterstaining with DAPI (Vector, CA, USA), mounting, and photography using a multiphoton laser scanning microscope (Zeiss Lsm 780 NLO, Germany). Experiments were replicated at least twice.

Western blotting

Proteins from various tissues (stomach, foregut, midgut, hindgut, head kidney, posterior kidney, muscle, liver, and serum) at different time points (0 h, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 90 min, 180 min, 360 min, 720 min, 1440 min) were extracted for Western blot analysis. For the serum samples, blood was collected from each fish via caudal vessel puncture. The samples were then centrifuged at 3000 g for 5 min to isolate the serum [54]. The detailed procedures for Western blot analysis, along with information on the experimental antibodies, can be found in the Supplementary Materials and Methods.

Enzyme-linked immunosorbent assays (ELISA)

Serum samples collected from red-spotted grouper in each feeding group underwent analysis for double-antibody sandwich ELISA. The detailed procedures for ELISA analysis, along with information on the experimental antibodies, can be found in the Supplementary Materials and Methods.

Commensal *bacteria* isolation

In September 2019, during a short-term feeding experiment of *E. akaara* in Ningde, Fujian Province, China, intestinal samples were aseptically collected from 10 individuals. The samples were then incubated aerobically at 28 °C for 24 h on Brian Heart Infusion (BHI, Oxoid, UK) plates and 2216E (Haibo Biological Co., Ltd, China) plates. Subsequently, the colonies were recovered and purified, and 2 dominant isolates were obtained from BHI and 2216E plates, respectively. These isolates were stored at − 80 °C in BHI and 2216E supplemented with 20% glycerol.

DNA sequencing, assembly, gene prediction and annotation of two isolated commensal *bacteria*

Detailed procedures of DNA sequencing and assembly, gene prediction and annotation of *B. thuringiensis* and *V. harveyi* are provided in Supplementary Materials and Methods.

Transmission electron microscope

Transmission electron microscope observation utilized BHI as the growth medium for *B. thuringiensis* and 2216E for *V. harveyi.* Ultrathin sections and negative staining were performed on *B. thuringiensis* and *V. harveyi* following standard protocols [55], followed by further observation with a transmission electron microscopy (FEI Tecnai G2 F20). Experiments were repeated at least twice.

Electron microscope ultramicroscopy assay

Morphological changes of bacterial cells in the presence of Scy-hepc as observed by scanning electron microscopy following our previous research [56]. Bacteria in the logarithmic growth phase were collected and suspended in NaPB buffer to a concentration of approximately $10^{\wedge 7}$ CFU/mL. A final concentration of 48 μM Scy-hepc was coincubated with the bacteria at 37 °C for 30 min. The bacteria were then harvested by centrifugation (5000 g, 5 min), resuspended in 2.5% (v/v) glutaraldehyde (Sigma, Germany) fixative, and incubated at 4 °C overnight. After three washes, 10 μL of NaPB buffer was added to obtain a concentrated suspension. This suspension was then added dropwise onto pre-cut poly-L-lysine-coated slides and placed on ice for 30 min. Excess liquid was removed using filter paper, and the samples were dehydrated in an ethanol gradient before being critical point dried (EM CPD300, Leica, Germany). The samples were then coated with metal using an ion sputter coater (JFC-1600, Jeol, Germany) and observed with a scanning electron microscope (SEM, Zeiss SUPRA 55, Germany).

Motility assays

Surfing, swimming, and swarming assays were conducted on BHI and 2216E medium. The surfing assay involved 0.3% (w/v) agar with 0.4% (w/v) mucin, while the swimming assay had no mucin, and the swarming assay used 0.7% (w/v) agar. Scy-hepc (48 μM), polymyxin E (2 μg/mL) and control (sterile water) were added dropwise to the center of the plates and dried. *B. thuringiensis* and *V. harveyi* were subcultured at a 1:100 ratio in liquid LB medium and cultured until reaching an absorbance 0.4 to 0.5 at an optical density of 600 nm. Subsequently, 1 μL of the culture was inoculated onto plates and incubated for 24 h at 37 °C. The visible growth zones were measured hourly at 37 °C over a 24 h period in the incubator to ensure continuous incubation. Notches were marked at the endpoints of the motility zones at each time point to ensure consistent measurements from a fixed side of the motility colony. Experiments were repeated at least twice.

qRT-PCR analysis

Scy-hepc (48 μM), polymyxin E (2 μg/mL) and control (sterile water) were co-incubated with *B. thuringiensis* and *V. harveyi* for 6 h, 12 h and 24 h to examine the effect of Scy-hepc on motility and flagellar assembly of the commensal bacteria. qRT-PCR was employed to determine the expression levels of genes associated with flagellar assembly and virulence. The templates for qRT-PCR were prepared using RNA samples independently isolated from three biological replicates Specific primers were designed based on the corresponding sequences in the genomes of *B. thuringiensis* and *V. harveyi* (Table S8). The comparative CT method (2-ΔΔCT method) was employed for analyzing expression levels [24]. qRT-PCR was conducted using an Applied Biosystems 7500 instrument (Life Technologies, USA), and the resulting data were analyzed using the 7500 System SDS Software. Experiments were repeated at least twice. Data are expressed as means ± SD (n = 6). Significant differences between two groups were determined using a two-tailed Student's t-test, while comparisons involving more than two groups employed a one-way ANOVA. *P* value of < 0.05 was considered significant difference.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-024-00342-3.

Additional file1.

Additional file2.

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Author contributions
HS, LW and KW designed the study. HS performed the experiments and analyzed data. HS and LW drafted manuscript and figures. XM provided the isolation of commensal bacteria and analyzed data. HS, WZ, HP, HH, HC provided the fish farming and sample collection. KW contributed all of reagents, materials and analysis tools. FC and KW were in charge of the funding acquisiton. LW, FC and KW were in charge of the supervision and correction of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
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 PRJNA1026366. And the reviewer link is https://dataview.ncbi.nlm.nih.gov/object/PRJNA1026366?reviewer=fn9ifuqtu6sc4hov5c13fj8rf7.

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 (XMULAC20240211).

Consent for publication
Not applicable.

Competing interests
No conflict of interest exists in the submission of manuscript, and the authors declare that they have no competing interests.

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