Comparative Analyses of Scylla olivacea Gut Microbiota Composition and Function Suggest the Capacity for Polyunsaturated Fatty Acid Biosynthesis

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Abstract
Although numerous studies in aquatic organisms have linked lipid metabolism with intestinal bacterial structure, the possibility of the gut microbiota participating in the biosynthesis of beneficial long-chain polyunsaturated fatty acid (LC-PUFA) remains vague. We profiled the gut microbiota of the mud crab Scylla olivacea fed with either a LC-PUFA rich (FO) or a LC-PUFA-poor but C18-PUFA substrate-rich (LOCO) diet. Additionally, a diet with a similar profile as LOCO but with the inclusion of an antibiotic, oxolinic acid (LOCOAB), was also used to further demarcate the possibility of LC-PUFA biosynthesis in gut microbiota. Compared to diet FO treatment, crabs fed diet LOCO contained a higher proportion of Proteobacteria, notably two known taxonomy groups with PUFA biosynthesis capacity, Vibrio and Shewanella. Annotation of metagenomic datasets also revealed enrichment in the KEGG pathway of unsaturated fatty acid biosynthesis and polyketide synthase-like system sequences with this diet. Intriguingly, diet LOCOAB impeded the presence of Vibrio and Shewanella and with it, the function of unsaturated fatty acid biosynthesis. However, there was an increase in the function of short-chain fatty acid production, accompanied by a shift towards the abundance of phyla Bacteroidota and Spirochaetota. Collectively, these results exemplified bacterial communities and their corresponding PUFA biosynthesis pathways in the microbiota of an aquatic crustacean species.

Keywords Gut microbiota · Metagenomics · Mud crab · Scylla olivacea · Polyunsaturated fatty acid

Introduction
Long-chain polyunsaturated fatty acids (LC-PUFA), including eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), and arachidonic acid (ARA; 20:4n-6), are essential for proper cellular, neuronal, cardiovascular, and anti-inflammatory activities [1]. While humans can biosynthesize LC-PUFA from the C18 polyunsaturated fatty acids (PUFA), there is still a need to rely on dietary intake of aquatic food products to fulfill the physiological requirements for n-3 LC-PUFA [2, 3]. The biosynthesis of LC-PUFA requires the fatty acyl desaturases (Fads) and elongase of very long-chain fatty acid (Elovl) enzymes to desaturate and elongate the fatty acyl chain, respectively [4]. The capacity for LC-PUFA biosynthesis within a species is dependent on the Fads/Elovl genetic machinery [5]. In natural habitats, an incomplete LC-PUFA biosynthesis pathway could possibly be compensated by selective feeding of prey with higher LC-PUFA content or genetic modification of...
LC-PUFA biosynthesis enzymes to enhance biosynthesis [6, 7].

Among the invertebrates, the extent of de novo LC-PUFA biosynthesis in crustaceans is still unclear due to the vast diversity of this phylum [8, 9]. A complete repertoire of Fads and Elovl fulfilling all the necessary steps for LC-PUFA biosynthesis was only recently shown in a harpacticoid copepod, Tigriopus californicus [10]. As for decapods, functional Elovl has been reported from the orange mud crab, Scylla olivacea [11, 12], and swimming crab Portunus trituberculatus [13]. However, the actual existence of functional Fads remains contentious [9]. The Scylla mud crabs have huge ecological and economical importance in mangroves of the Indo-Pacific region [14]. As aquaculture candidate species, Scylla crabs possess fast growth rate, large size, high reproductive capacity, and adaptability to different farming systems. In many countries, Scylla farming still depends on freshly prepared wet feed [15]. Knowledge on the capacity for LC-PUFA biosynthesis in Scylla will facilitate the formulation of effective formulated feeds.

The gut microbiome is integral for the overall gut function, immunity, and nutrient processing [16]. The relationship between gut microbiota and various aspects of lipid metabolism such as deposition, digestibility, accumulation, adsorption, synthesis, and obesity have been investigated [17, 18]. Studies in decapods have shown the influence of different dietary fatty acids on gut microbiota profile [19–21]. There is a complex interplay between host dietary lipid intake, gut microbiota characteristics, and tissue fatty acid composition [22]. It is increasingly recognized that PUFA-synthesizing bacteria genera are present in the microbiota of aquatic animals [23, 24]. While decapods can rely on endogenous biosynthesis activities and/or exogenous dietary intake for the supply of LC-PUFA, it is not inconceivable that the host gut microbiota may play a requisite role.

In view of our interest to unravel a potential role in LC-PUFA biosynthesis within the mud crab gut microbiota, a feeding trial was designed using experimental diets with limited or high levels of LC-PUFA for eight weeks. At the end of the trial, metagenome profiling of the gut microbiota and analysis of fatty acid composition of crab tissues were conducted. Overall, the variations in the dynamics of the gut microbiota of experimental animals provide insights into the involvement of bacterial groups with LC-PUFA biosynthesis function in S. olivacea.

**Material and Methods**

**Experimental Animals and Diets**

Fresh male crabs (weight 124±14.8 g; carapace length 9±0.3 cm) were purchased from local fishermen. Upon arrival, three crabs were dissected immediately to remove the digestive tract and designated as wild crabs (WT). The remaining crabs were acclimatized for two weeks and fed a standard commercial diet. Twenty-seven crabs in the intermolt stage were randomly distributed into recirculating glass aquariums (0.60×0.31×0.30 m) at density of one crab per tank. Water parameters were at 18–24 ppt salinity, 25–29 °C, pH 7.5–8.3, and dissolved oxygen > 5 mg/L, with 80% water change every 48 h.

Three isonitrogenous and isolipidic diets were formulated (Table S1). Diet FO utilized fish oil (FO), whereas LOCO used a 1:1 blend of linseed oil and corn oil as primary lipid sources. Diet LOCOAB had a similar formulation as LOCO, with the inclusion of 75 mg/kg oxolinic acid. This concentration was based on a preliminary PCR amplification of the 16S rRNA gene to quantify bacterial load [25] (Fig. S1). The fatty acid composition of the three diets reflects their dietary lipid source, with diet FO enriched in LC-PUFA and diets LOCO and LOCOAB enriched in C18 PUFA but devoid of LC-PUFA (Table S2). Nine replicate tanks were allocated to each experimental diet. The crabs were fed twice daily till satiation for eight weeks.

**Sample Collection and DNA Extraction**

At the end of the trial, three individual crabs were randomly collected for DNA extraction from each dietary treatment. Crabs were euthanized at −20 °C for 20 min, washed, and disinfected with 75% ethanol. Digestive tracts were aseptically removed, with gut contents and intestinal mucosa harvested into sterile tubes. Genomic DNA was extracted from each individual sample as biological replicate using the DNeasy PowerSoil kit (Qiagen, Germany). The quality and quantity of isolated DNA were assessed using electrophoresis and NanoDrop spectrophotometer (Thermofisher Scientific, USA).

**16S rRNA Amplicon Sequencing and Analyses**

The V3–V4 variable region of the 16S rRNA gene was amplified using the primers 341F (5′-CCTACGGGNGGC WGCAG-3′) and 806R (5′-GACTACHVGGGTWT CTAAT-3′). Libraries for 16S rRNA sequencing were prepared as described previously [26]. Sequencing was performed on an Illumina MiSeq platform, generating 250-bp paired-end reads. The sequencing data were analyzed using the QIIME pipeline (v1.9.1) [27]. Raw sequencing data were pre-processed using Trimmomatic (v0.36) to remove adapter sequences and low-quality bases. Paired-end reads were merged using FLASH, and chimeric sequences were removed using the UCHIME algorithm. Sequences were clustered into operational taxonomic units (OTU) using UPARSE (v7.1) with a 97% similarity.
cut-off. The representative sequences from each OTU were taxonomically classified against the SILVA database [28] using Ribosomal Database Project classifier (v2.2) with a 70% confidence threshold. Alpha diversity was estimated using richness (Chao1 index) and diversity (Simpson and Shannon index) parameters. Non-metric multidimensional scaling (NMDS) of the Bray-Curtis distance and principal coordinate analysis (PCoA) of the weighted UniFrac distance was applied to analyze the beta diversity. An unweighted pair group method with arithmetic mean tree was constructed using QIIME. Venn diagram, species accumulation, and rank abundance curves were generated using the R project for statistical computing. A linear discriminant analysis (LDA) effect size (LEfSe) was performed to present the enrichment of bacterial taxa between groups [29].

**Shotgun Metagenomic Sequencing and Analyses**

Sequencing libraries were prepared using the Illumina TruSeq Nano DNA library preparation kit and sequencing was performed on an Illumina NovaSeq 6000 platform at 150-bp paired-end. The raw sequencing data were processed to filter low-quality reads and adapters using Trimmomatic. Taxonomic classification by k-mer-based lowest common ancestor approach was performed using Kraken2 (v2.0.6). De novo assembly of the clean reads was carried out using Megahit (v1.1.1) [30] with minimum contig length set to 500 bp. The genome of a closely related species, *Portunus trituberculatus* [31], was used as reference sequence to remove host contigs from the metagenome assemblies. Open reading frames (ORF) were predicted using MetaProdigal (v2.6.3), and the ORF were clustered using CD-HIT (v4.8.1) at 95% identity and 90% coverage. Annotation of the unique gene set was performed through BLASTp against the NCBI non-redundant protein (NR), Clusters of Orthologous Groups, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases at an e-value threshold of 1e-5. Antibiotic resistance genes in the metagenomes were predicted by a search against the Comprehensive Antibiotic Resistance Database [32]. The hits to keto-acyl synthase (KS) sequences were retrieved from BLASTp of the unique gene set to the NCBI NR database and aligned using MUSCLE, followed by trimming with trimAl (v1.2). The best model for the amino acid alignment was evaluated using ProTest (v3.4.2) based on Akaike criterion. Maximum likelihood analysis of KS utilized RAxML (v8.2.12) [33] with 1000 bootstraps and GAMMA + VT model. The phylogenetic tree was illustrated with the Interactive Tree of Life (http://itol.embl.de).

**Fatty Acid Extraction and Composition Analysis**

Total lipids were extracted from muscle and hepatopancreas of individual biological replicate via sonification in 2:1 (v/v) chloroform:methanol. Trans-esterification of fatty acids to fatty acid methyl esters (FAME) was as previously described [12]. Quantification of FAME was carried out with a GC–MS QP2010 Ultra gas chromatograph mass spectrometer (Shimadzu, USA), fitted with a Supelco 2380 high-polarity-fused silica capillary column (30 m length, 0.25 mm inner diameter, and 0.20 μm film thickness; SGE, USA). Gas, temperature changes of the GC–MS, identification of individual FAME, and conversion to composition of fatty acids calculation were as described elsewhere [11]. One-way analysis of variance (ANOVA) and Tukey’s post hoc test (P < 0.05) were used to determine the differences between the three different dietary treatments (n = 3) for tissue fatty acid composition.

**Results**

**Crab Survival and Growth**

There was no mortality in dietary treatments of FO and LOCO throughout the feeding trial (Table 1). Diet LOCOAB resulted in 77% survival, lower percentage of molting, and specific growth rate as compared to diets FO and LOCO. Higher feed conversion ratio was also observed with LOCOAB treatment, despite similar feed intake with crabs from the other dietary treatment.

**Overview of the Metagenomic Sequencing Data**

The 16S rRNA amplicon sequencing produced 2.95 million reads from the WT, FO, LOCO, and LOCOAB samples, ranging from 212,812 to 292,709 reads for each sample (Table S3). These sequences were delineated into 18,514 OTU, corresponding to an average of 1543 OTU per sample. A total of 559 million paired-end reads, with an average of 47 million reads per sample, were generated from shotgun metagenomic sequencing (Table 2). After trimming, 537 million clean reads were assembled into 52,275–85,353 OTU, corresponding to an average of 1543 OTU per sample. A total of 559 million paired-end reads, with an average of 47 million reads per sample, were generated from shotgun metagenomic sequencing (Table 2). After trimming, 537 million clean reads were assembled into 52,275–85,353 contigs with a total length of 94–174 Mb and N50 contig length of 2,615–5,843 bp. A non-redundant protein-coding gene set containing ~1.89 million ORF was predicted from the 12 metagenomes.

For community richness and diversity comparison, alpha diversity parameters were calculated from the proportion of OTU. The Chao 1 index, an indicator of microbiota community richness, varied from 1,410 to 2,124. Among the different treatments, FO and LOCO samples exhibited higher OTU and Chao1 index values than WT and LOCOAB.
Similarly, Shannon index value was also highest in FO. The Good’s coverage estimator of sequencing completeness ranged from 0.998 to 0.999, indicating high species coverage within the samples. Additionally, the species accumulation curve appeared to flatten after 8, indicating that the number of samples adequately reflects the species abundance (Fig. S2A). LOCO samples showed the widest and smoothest curve in rank abundance analysis, indicating the highest species richness and, concomitantly, uniformity among sample groups (Fig. S2B). Samples LOCOAB resulted in the lowest species richness and community evenness.

The PCoA and NMDS analyses of beta-diversity were performed to visualize the differences in bacterial communities among samples (Fig. 1A and 1B). Principal component 1 (PC1) and PC2 accounted for 72.34% and 17.38% of the composition variance, respectively, reflecting the dissimilarity in the bacterial community composition among different dietary groups. Additionally, the hierarchical clustering tree showed a clear distinction among these groups while the replicates within each group are consistently grouped (Fig. 1C). The Venn diagram showed 684 shared OTU among the four groups, and 389, 133, 165, and 153 unique OTU within the WT, FO, LOCO, and LOCOAB groups, respectively (Fig. 1D). Treatments FO and LOCO shared the highest number of OTU (1,762), followed by WT and FO (1,245), WT and LOCO (1,231), and lastly, LOCO and LOCOAB (1,166).

**Gut Microbiota Taxonomic Composition**

At the phylum level, Proteobacteria (21.8–57.4%), Firmicutes (11.8–38.8%), Bacteroidota (2.7–32.0%), Fusobacteriota (1.3–11.1%), and Tenericutes (0.3–52.2%) were the core groups in all sample types, accounting for 86.2% of the total reads (Fig. 2A and 2B). The phylum Tenericutes was reclassified into the Bacilli class of Firmicutes following the SILVA database and, therefore, not annotated in the 16S

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**Table 1** Growth parameters of *S. olivacea* fed the experimental diets after eight feeding weeks

| Nutritional indices                  | Experimental diets |
|--------------------------------------|--------------------|
|                                      | FO                 | LOCO               | LOCOAB              |
| Initial weight (g)                   | 126.0±19.52        | 119.7±16.07        | 125.3±14.19         |
| Final weight (g)                     | 183.3±34.53        | 166.3±24.58        | 153.3±30.06         |
| Weight gain (%)<sup>1</sup>          | 45.4±12.12         | 38.8±2.39          | 9.0±10.15<sup>a</sup> |
| SGR (%/day)<sup>2</sup>              | 0.7±0.15           | 0.8±0.31<sup>a</sup>| 0.3±0.23<sup>a</sup>  |
| Initial carapace width (cm)          | 8.6±0.58           | 8.7±0.31           | 8.9±0.32            |
| Final carapace width (cm)            | 10.5±0.38          | 10.5±0.55          | 9.6±1.01            |
| FCR<sup>3</sup>                      | 1.8±0.29<sup>a</sup>| 2.0±0.98           | 11.5±8.54<sup>b</sup> |
| Survival (%)<sup>4</sup>             | 100%               | 100%               | 77%                 |
| Molting (%)<sup>5</sup>              | 89%                | 89%                | 22%                 |

The results are presented as the means ± SEM (n = 3). The results are represented by mean ± SEM (n = 9). Different alphabets denote significant differences among dietary treatments as determined by one-way ANOVA followed by Tukey’s HSD test (P < 0.05).

1. Weight gain (%) = 100 × [(WF − WI)/WI], where WF is final individual crab weight (g) and WI is initial individual crab weight (g).
2. SGR, specific growth rate = 100 × ([ln(WF) − ln(WI))/days], where WI is initial individual crab weight (g) and WF is final individual crab weight (g).
3. FCR, feed conversion ratio = individual dry feed intake (g)/individual wet weight gain (g).
4. Survival (%) = 100 × (final number of surviving crabs/initial number of crabs).
5. Molting (%) = 100 × (final number of molted crabs/initial number of crabs).

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rRNA amplicon analysis. The WT microbiota samples were dominated by Firmicutes and Tenericutes, while in FO, Proteobacteria and Fusobacteria were the most abundant. Proteobacteria also dominated the LOCO microbiota, while for diet LOCOAB treatment, Bacteroidota, Proteobacteria, and Spirochaetota were most abundant. Experimental diet treatment reduced the abundance of Firmicutes and Tenericutes, increasing the proportion of Proteobacteria, Fusobacteria, Bacteroidota, Spirochaetota, and Campylobacterota, respectively. The microbiota of LOCO treatment showed higher abundance of Proteobacteria, Firmicutes, and Tenericutes, while the abundance of Bacteroidota, Fusobacteria, Spirochaetota, and Campylobacterota were reduced compared to diet FO. Treatment with diet LOCOAB lowered the abundance of Proteobacteria, Firmicutes, Tenericutes, and Fusobacteria, while Bacteroidota and Spirochaetota increased.

The Proteobacteria phylum was mainly represented by Gammaproteobacteria, predominantly from the Vibrionaceae family (Fig. S3 and S4). Fusobacteria in the FO microbiota was represented by the Fusobacteriaceae family from Fusobacteriia class. In WT samples, the Entomoplasmatales Incertae Sedis family of Bacilli class was predominant in Firmicutes, and the Mycoplasmataceae family of Mollicutes class was most abundant among Tenericutes. Within the Bacteroidota, the Marinilabilaceae and Prolixibacteraceae families dominated the LOCOAB microbiota. At the genus level, both the 16S and shotgun datasets showed the dominance of Candidatus Hepatoplasma in the WT microbiota (Fig. 2C and 2D). Compared to wild crabs, there was a reduction in Candidatus Hepatoplasma, alongside Mycoplasma, Photobacterium, Spiroplasma, Paraclostridium, Bacillus, and Hypnocyclicus in the microbiota of experimentally fed animals. In contrast, the abundance of Vibrio, Sediminispirochaeta, Sunxiuqinia, Ruegeria, Arcobacter, Malaciobacter, and Carboxylicivirga was elevated. In diet LOCO, Vibrio, Candidatus Hepatoplasma, Shewanella, and Ferrimonas showed higher abundance than diet FO, while the abundance of Sediminispirochaeta, Carboxylicivirga, Sunxiuqinia, Psychrilyobacter, Arcobacter, Malaciobacter, Propionigenium, and Halarchobacter was lower. In LOCOAB samples, genera such as Vibrio, Candidatus Hepatoplasma, Ruegeria, Shewanella, Mycoplasma, Spiroplasma, Photobacterium, and Psychrilyobacter were reduced, while Sediminispirochaeta, Carboxylicivirga, Sunxiuqinia, and Oceanispirochaeta were elevated (Fig. S5).

Supervised comparisons using LEfSe (LDA > 4.0) identified 50 differentially abundant taxa ranging from the phylum to OTU level (12 in WT; 16 in FO; 7 in LOCO; 15 in LOCOAB) among the dietary treatments (Fig. 3).
Fig. 2  The composition of gut microbiota in *S. olivacea* from wild crabs (WT); fish oil (FO); linseed oil and corn oil (LOCO); and linseed oil, corn oil, and oxolinic acid (LOCOAB) samples. Phyla analyzed by (A) 16S rRNA amplicon and (B) shotgun metagenomic sequencing. Genera analyzed by (C) 16S amplicon and (D) shotgun sequencing. Phyla with percentage value of less than 0.1% are included in “Others”, and only the top 15 highly abundant genera are shown.
**S. olivacea** Gut Microbiota Function

A total of 84.4% of the unique gene sets from all samples were assigned into functional groups (Table S4). These genes were further annotated to 44 pathways at KEGG level 2, with an abundance trend of the highest enrichment in global and overview maps (25.4%) > carbohydrate metabolism (8.3%) > energy metabolism (5.2%) (Fig. S6). The relative composition of these three categories at KEGG level 3 is illustrated in Fig. S7.

A heatmap comparing the enriched level 2 categories between the four sample groups revealed a higher relative abundance of nucleotide metabolism, transcription, translation, folding, sorting and degradation, replication and repair, and signaling molecules and interaction pathways in WT microbiota (Fig. 4A). The microbiota of crabs fed FO, LOCO, or LOCOAB were enriched in global and overview maps, numerous metabolisms including lipid, amino acid, other amino acids, cofactors and vitamins, terpenoids and polyketides, and biosynthesis of secondary metabolites. Among the diets, LOCO enriched the cell motility, cellular community-prokaryotes, membrane transport, signal transduction, and xenobiotics degradation and metabolism.
functions. For diet LOCOAB, highest abundance of global and overview maps and metabolisms of major nutrients were obtained. The mapping of the *S. olivacea* gut metagenomes against the Comprehensive Antibiotic Resistance Database recovered antibiotic resistance genes from 15 main families, corresponding to seven drug mechanisms (Fig. S8).
In relevance to the main objective of this study, a heatmap illustration to compare the KEGG level 3 lipid metabolism categories between dietary treatments was built (Fig. 4B). Compared to WT animals, crabs fed the respective experimental diet enriched various lipid metabolism pathways, including fatty acid biosynthesis and fatty acid degradation. Compared to diet FO, pathways on fatty acid degradation, synthesis and degradation of ketone bodies, glycerophospholipid metabolism, ether lipid metabolism, linoleic acid metabolism, arachidonic acid metabolism, and biosynthesis of unsaturated fatty acids were enriched in the LOCO microbiota. In LOCOAB microbiota, these functions were impeded, although an increase in sphingolipid metabolism, steroid hormone biosynthesis, fatty acid elongation, and fatty acid biosynthesis were observed. In terms of bacteria taxa, the lipid metabolism function were mostly contributed by Proteobacteria and represented by the families Vibrionaceae, Rhodobacteraceae, and Shewanellaceae (Fig. 5A). At the genus level, lipid metabolism in WT microbiota was contributed by Vibrio, Shewanella, and Clostridium (Fig. 5B). In diet LOCO, the Ruegeria and Shewanella genera contributed higher towards lipid metabolism as compared to diet FO. While diet LOCOAB did not affect the contribution levels of these two genera, there was an increase in contribution from Vibrio, Arcobacter, Clostridium, and Bacteroides.

Numerous genes encoding for the fatty acid biosynthesis and biosynthesis of unsaturated fatty acids enzymes were annotated from the microbiota samples (Fig. S9 and S10). Additionally, there was an increase in the abundance of several sequences assigned to the KEGG function biosynthesis and metabolism of fatty acids pathways.
of unsaturated fatty acids in diet LOCO treatment. Among these are acyl-CoA oxidase, stearoyl-CoA desaturase, and acyl-coenzyme A thioesterases, which were also impeded in diet LOCOAB (Table S5). A higher abundance of fatty acid biosynthesis genes was observed in the microbiota of LOCOAB treatment. The complete pathways for acetate and butyrate formation and a partial propionate pathway are present in *S. olivacea* gut microbiota (Fig. S11–S13). Heatmap analysis showed that LOCOAB treatment increased the function of short-chain fatty acids (SCFAs) production as compared to LOCO treatment (Fig. 4C).

Several marine species, in particular *Shewanella* and *Vibrio*, can synthesize PUFA de novo through an anaerobic route, using the fatty acid synthase/polyketide synthase (FAS/PKS)-like enzyme system [34, 35]. The gene cluster encoding this system consists of four ORF, represented by the *pfaABCD* genes [36]. Since this pathway is not included in the KEGG database, we examined the *S. olivacea* gut shotgun metagenomic datasets for the diversity and distribution of the keto-acyl synthase (KS) domain harbored within the *pfaA* homolog. The KS sequences recovered in this study form a large monophyletic clade with known KS from *Vibrio*, *Shewanella*, and *Photobacterium*, showing conservation of the KS domain across species (Fig. 6). A total of 362 KS sequences were recovered, with 36, 135, 127, and 64 sequences from WT, FO, LOCO, and LOCOAB samples, respectively. Therefore, in contrast to SCFA pathways, the inclusion of oxolinic acid impeded the PKS pathway in *S. olivacea*.

In hepatopancreas, the highest levels of DHA were obtained with diet FO (Table S6). Diet LOCO resulted in the deposition of α-linolenic acid (ALA), linoleic acid (LA), and several intermediates of the LC-PUFA biosynthesis pathway such as 18:4n-3 and 22:5n-3, which implies some degree of LC-PUFA biosynthesis. Interestingly, the percentage of ALA and LA were significantly higher in diet LOCOAB despite this diet having the same lipid level composition with diet LOCO. Crabs fed diet FO expectedly have the highest deposition of LC-PUFA in muscle tissue. There was no significant difference in LC-PUFA levels between diet LOCO and LOCOAB.

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**Fig. 6** Maximum likelihood phylogenetic tree of keto-acyl synthase protein sequences inferred using RAxML (GAMMA + VT model, 1000 bootstraps). The *Saccharopolyspora erythraea* EryAI sequence was used as the outgroup. Sequences from WT, FO, LOCO, and LOCOAB samples are indicated by red, blue, orange, and green strips, respectively. Reference strain sequences are shown in bold and bootstrap values ≥ 90 are indicated by dots.
Discussion

Regardless of treatment, Proteobacteria, Firmicutes, Bacteroidota, Fusobacteriota, and Tenericutes are the main phyla in *Scylla olivacea* gut microbiota, paralleling previous findings in *Scylla* crab and Chinese mitten crab [37–39]. As facultative or obligate anaerobes, the consumption of oxygen by Proteobacteria contributes to an anaerobic environment within the gut, facilitating the proliferation of strict anaerobes [40]. Firmicutes and Bacteroidota are known to participate in carbohydrate and polysaccharide digestion [19, 41]. Members of the Fusobacteriota were linked with vitamin synthesis and amino acid fermentation [42, 43]. The prevalence of Tenericutes in the vertebrate microbiota was linked to polysaccharide digestion [44, 45]. Similar to previous findings in crabs, the main bacteria groups in wild-caught *S. olivacea* are Tenericutes and Firmicutes [37, 46]. *Candidatus Hepatoplasma*, the predominant genus in the wild crabs in this study, is attributed to adaptation towards limited food availability in *S. paramamosain* [37]. In the isopod intestinal tract, this genus conferred a higher host survival rate during food scarcity [47]. We also observed lower proportions of Firmicutes, Tenericutes, and *Candidatus Hepatoplasma* in crabs maintained by the experimental diets. From a dietary perspective, this alteration could be due to a higher intake of dietary lipid as compared to the wild-caught crabs.

Our results showed that high dietary n-3 LC-PUFA intake increased the diversity of *S. olivacea* gut microbiota. Elsewhere, the microbiota community in *S. paramamosain* and *P. vannamei* showed higher diversity with FO-based diet than vegetable oil (VO)-based diet treatment [19, 38, 48]. In the fish intestine, dietary n-3 LC-PUFA was reported to reverse microbial dysbiosis, allowing the proliferation of healthy bacteria [49, 50]. Higher diversity was also reported in gut microbiota of transgenic animals [19, 38, 48]. An alternative LC-PUFA biosynthesis pathway, termed as the PKS-like system, was first discovered in marine microorganisms and subsequently reported to be widespread in prokaryotes from various niches, including intestinal microbiota [24, 34, 62]. Significant hits matching the KS domain were also obtained from FO- or LOCO-fed *S. olivacea* microbiota datasets, indicating an enrichment of the PKS pathway. The number of KS sequences detected in our samples is noticeably higher than those obtained from environmental metagenomes [63], which indicates aquatic animal gut as hotspots for LC-PUFA biosynthesis. The higher abundance of genes encodings for the PKS pathway function in diet LOCO treatment could be associated with the higher composition of
**Vibrio** and **Shewanella**, two known genera with the capacity for EPA and DHA biosynthesis through the PKS system [34, 35]. Taken together, our results demonstrated elevated abundance in genes related to various pathways for LC-PUFA biosynthesis in **S. olivacea** LOCO gut microbiota.

Oxolinic acid, a broad-spectrum antibiotic commonly used in aquaculture [64], was utilized in this study as a non-invasive and unbiased approach to possibly impede the proliferation and function of gut bacteria with potential role in LC-PUFA biosynthesis. A similar approach was used to limit the contribution of microbiota bacterial groups with speculated roles in lipid metabolism [24, 61]. Consistent with previous findings, we observed a reduction in gut microbiota diversity in **S. olivacea** fed diet LOCOAB [24, 65]. The abundance of Proteobacteria was also reduced, caused mainly by a decrease in **Vibrio**. In contrast, phyla Spirochaetota and Bacteroidota, mainly represented by **Sediminispirochaeta, Carboxylicivirga**, and **Sunxiuginia**, showed increased abundance. Function wise, the inclusion of oxolinic acid reduced gene sets from several lipid metabolism pathways, including biosynthesis of unsaturated fatty acids and PKS pathway. However, higher abundance of sequences for synthesis of SCFAs including acetate was observed. This increase could be associated with the abundance of Bacteroidota, a known acetate producer in the microbiota [66].

In Chinese mitten crab, the gut microbial community influences the host fatty acid composition [67]. Elsewhere, PUFA-biosynthesizing zooxanthellae symbionts were shown to affect the LC-PUFA composition of their respective coral hosts [68, 69]. Therefore, we relate the compounding changes in structure and function of **S. olivacea** microbiota fed the different experimental diets to the respective hepatopancreas and muscle PUFA composition. We found lower LC-PUFA content in hepatopancreas and muscle of crab fed diet LOCO or LOCOAB as compared to diet FO. This reiterates previous findings in mud crabs fed VO-based diets [11, 70]. This implies that the LC-PUFA biosynthesis activities in crabs fed diet LOCO driven by the in vivo enzymatic capacity of **S. olivacea** and gut bacterial communities are unable to produce tissue deposition levels of LC-PUFA at levels comparable to diet FO. Interestingly, despite metagenome datasets showing a reduction in the composition of **Vibrio** and **Shewanella**, coupled with reduced unsaturated fatty acid biosynthesis activities in the microbiota of crab fed diet LOCOAB, there was no significant reduction in levels of muscle LC-PUFA when compared to the values of LOCO treatment. As aforementioned, there was an increase in the function of SCFA production within the microbiota of LOCOAB crab, which could be a source of LC-PUFA for the host. The possibility of representatives from the phylum Bacteroidota contributing to LC-PUFA biosynthesis could not be ruled out. Secondly, although the composition of Proteobacteria and **Vibrio** were reduced in the microbiota of LOCOAB treatment, these two groups still occupied a significant proportion of the overall microbiota diversity and, therefore, might still contribute in terms of LC-PUFA biosynthesis. In sesarmid crabs, antibiotic treatment did not result in a significantly lower level of DHA, presumably due to incomplete removal of gut bacteria [24]. The persistence of EPA and ARA in the muscle tissues of LOCOAB crabs could also be due to the lower utilization of these PUFAs as substrates for eicosanoid production. This reason is partially supported by the observed decrease in arachidonic acid metabolism in the metagenome dataset from LOCOAB. Lastly, the lack of molting observed in LOCOAB crabs during the whole feeding trial could also mean the conservation of LC-PUFA in tissues [71].

**Conclusion**

Overall, our study shed light on the LC-PUFA biosynthesis activities within the gut microbiota of **S. olivacea**. The microbiota of crabs with high C18-PUFA and limited LC-PUFA dietary intake showed an increase in **Vibrio** and **Shewanella**, in tandem with an increase in unsaturated fatty acid biosynthesis and PKS-like function. Additionally, the use of an antibiotic-supplemented experimental feed unravels the persistence of Spirochaetota and Bacteroidota, alongside elevated abundance of sequences for SCFAs synthesis, which could be another path for PUFA biosynthesis. Taken together, our study delineates potential bacterial taxonomy groups and functional pathways related to LC-PUFA biosynthesis in microbiota for the first time in an aquatic species.

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**Data Availability** The raw sequence data have been deposited in NCBI Sequence Read Archive under BioProject accession PRJNA737307. The metagenome assemblies have been deposited in NCBI under the same BioProject accession.

**Declarations**

**Conflict of Interest** The authors declare no competing interests.

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