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Transcriptome Analysis of the Marine Nematode *Litoditis marina* in a Chemically Defined Food Environment with Stearic Acid Supplementation

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Abstract: Stearic acid represents one of the most abundant fatty acids in the Western diet and profoundly regulates health and diseases of animals and human beings. We previously showed that stearic acid supplementation promoted development of the terrestrial model nematode *Caenorhabditis elegans* in chemically defined CeMM food environment. However, whether stearic acid regulates development of other nematodes remains unknown. Here, we found that dietary supplementation with stearic acid could promote the development of the marine nematode *Litoditis marina*, belonging to the same family as *C. elegans*, indicating the conserved roles of stearic acid in developmental regulation. We further employed transcriptome analysis to analyze genome-wide transcriptional signatures of *L. marina* with dietary stearic acid supplementation. We found that stearic acid might promote development of *L. marina* via upregulation of the expression of genes involved in aminoacyl-tRNA biosynthesis, translation initiation and elongation, ribosome biogenesis, and transmembrane transport. In addition, we observed that the expression of neuronal signaling-related genes was decreased. This study provided important insights into how a single fatty acid stearic acid regulates development of marine nematode, and further studies with CRISPR genome editing will facilitate demonstrating the molecular mechanisms underlying how a single metabolite regulates animal development and health.

Keywords: marine nematode; stearic acid; CeMM; development; transcriptome; protein translation; neuronal signaling

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1. Introduction

Stearic acid (SA, C18:0) is a saturated long-chain fatty acid (FA) and one of the most abundant fatty acids in the Western diet [1,2]. Unlike palmitic acid (PA, C16:0), dietary stearic acid does not increase atherosclerosis risk, while stearic acid was reported to reduce LDL (low density lipoprotein) cholesterol in humans [3–6]. Studies have shown that elevated circulating stearic acid lipids levels are related to lower blood pressure [7], improved cardiac function [8], and reduced cancer risk [9–13], thus, stearic acid seems to benefit human health differently from other saturated fatty acids. In addition, recent reports showed that stearic acid ingestion promotes mitochondrial fusion and increases fatty acid β-oxidation [14,15].

Modern foods are composed of many metabolites and it is critical to know which are the most important ones to our health. Since human diets are very complex and it is hard to delineate how a single metabolite regulates the development, reproduction, and lifespan of

the host, we have applied an axenic chemically defined diet CeMM (*C. elegans* maintenance medium) and the widely used biomedical model animal *Caenorhabditis elegans* to study how nutrition regulates animal development and health [16,17]. CeMM excludes variables associated with bacterial metabolism in the bacterial diet, making CeMM promising for use in drug screening [18–20]. In addition, the advantages of axenic cultivation and less need for astronaut intervention make CeMM widely used in the study of the effects of space flight on the development and physiology of tested animals such as *C. elegans* [21,22]. Given the composition and the amount of each component that can be manipulated in this chemically defined CeMM medium, it allows to study the precise mechanisms underlying how a single metabolite regulates animal health. We previously showed that dietary supplementation of the single fatty acid stearic acid significantly promotes development of *C. elegans* in a CeMM food environment [23]. However, whether stearic acid regulates the development of other nematodes and what the underlying mechanisms are remain unknown.

Litoditis marina is a free-living marine nematode, playing an essential role in marine ecosystems [24,25]. *L. marina* belongs to the same family as *C. elegans*, and has the advantages of short generation time, convenient laboratory culture, and its genome has been sequenced and annotated recently, making it a potential marine model animal [25,26].

In this study, we formulated sea-salt-CeMM that can support the growth of marine nematode *L. marina* and found that dietary supplementation with stearic acid could promote development of *L. marina*. We further employed RNA sequencing (RNA-seq) analysis to detect genome-wide transcriptional signatures of *L. marina* with dietary stearic acid supplementation. We found that stearic acid supplementation resulted in upregulation of the expression of genes involved in aminoacyl-tRNA biosynthesis, translation initiation and elongation, ribosome biogenesis, and transmembrane transporter in *L. marina*, whereas the expression of neuronal signaling-related genes was significantly downregulated with stearic acid supplementation. Our results provide clues for further research on the mechanisms underlying how a single metabolite regulates animal development and health.

2. Materials and Methods

2.1. Worms

The marine nematode *L. marina* wild strain HQ1 was isolated from intertidal sediments of Huiquan Bay, Qingdao. *L. marina* nematodes were cultured on seawater-NGM (SW-NGM) agar plates seeded with *Escherichia coli* OP50 as a food source [25,27]. Until this study, the *L. marina* nematodes had been maintained and propagated in the laboratory at 20 °C for about four years.

2.2. Sea-Salt-CeMM Preparation

The salinity of normal CeMM is too low to support the development of *L. marina* into adults. A CeMM medium supplemented with a final concentration of 15‰ sea-salt could support the growth and reproduction of *L. marina*. This sea-salt-CeMM medium was formulated as follows: 2× CeMM stock media was prepared as reported previously [16,23]. Then, the 15‰ sea-salt-CeMM plate was made by diluting the 2× CeMM stock to 1× via adding an equal volume of molten 3.4% agarose solution containing 30‰ sea-salt.

2.3. Dietary Stearic Acid Supplementation

Stearic acid (manufacturer: Aladdin) was prepared as 40 mM stocks in DMSO. For growth phenotype analysis, 100 µL of 2.5% solvent DMSO or 1 mM stearic acid suspension (prepared by diluting a 40 mM stock solution with sterile water) was added evenly on the surface of a 3.5 cm CeMM plate, and then dried in a dark incubator at 20 °C for one day before the experiments. For the dietary stearic acid supplementation RNA-seq experiment, 667 µL of 2.5% solvent DMSO or 1 mM stearic acid suspension was added evenly on the surface of 9 cm CeMM plate, then the plate was dried in a dark incubator at 20 °C for two days before use.

2.4. Synchronization of L. marina

The eggs were treated with a drop of worm bleaching solution (20% bleach, 0.5 M NaOH) on an empty SW-NGM plate. These eggs were then incubated overnight to obtain small scale synchronized L1.

For sample preparation of transcriptome, large-scale L1 synchronization was performed as described previously [27]. Briefly, a large number of eggs were treated for a short time with worm bleaching solution to kill the bacteria, which were then washed twice with sterile seawater and incubated overnight in sterile seawater to hatch. Finally, large numbers of synchronized L1 larvae were obtained through filtration via 500 grid nylon filter with 25 mm mesh size.

2.5. RNA-Seq Analysis

Synchronized *L. marina* L1s were transferred to corresponding 9 cm diameter 15‰ sea-salt-CeMM agarose plates supplemented with DMSO or stearic acid. The treated worms were cultured at 20 °C for 2.5 h and collected by washing with M9 buffer. Then, the obtained samples were frozen immediately in liquid nitrogen. Each treatment was performed with three biological replicates.

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). Then, with three biological replicates for each treatment, a total of six RNA sequencing libraries were prepared with 1 μg RNA per library using NEBNext UltraTM RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Then, the sequencing libraries were sequenced on an Illumina platform.

Reads containing adaptors or poly-N and low-quality reads were removed to obtain clean data. The minimum of base score Q20 was over 99% and Q30 was over 93.6%. The clean data were mapped to reference genome [25] by Hisat2 (v2.0.5, with the default parameters) [28]. Gene expression levels were normalized with FPKM. The differentially expressed genes (DEGs) were analyzed by DESeq2 [29]. Fold change ≥ 1.3 and a false discovery rate (FDR) < 0.05 were applied. The adjusted *p*-values (*p*adj) were calculated according to Benjamini and Hochberg [30] to control the FDR. Gene ontology (GO) enrichment analysis was carried out by the GOseq R packages [31]. The statistical enrichment of DEGs in KEGG pathways was performed by KOBAS [32].

2.6. Quantitative Real-Time PCR (qPCR) Analysis

Ten genes of our interest were randomly selected for qPCR validation, including upregulated genes EVM0009296/*gars-1*, EVM0009790/*nars-1*, EVM0003025/*eif-6*, EVM0016791/*M01G5.3*, EVM0007932/*ZK795.3*, EVM0017539/*F14E5.1*, and EVM0007205/*mct-6* and downregulated genes EVM0014526/*daf-37*, EVM0003946/*frpr-1*, and EVM0009311/*gnrr-5*.

For each sample, 500 ng RNA was used for reverse transcription using a cDNA synthesis with gDNA Remover kit (Toyobo, FSQ-301), then the resulting cDNA was used for qPCR analysis performed on ABI QuantStudio 6 Flex system using SYBR Green (Toyobo, QPK-201). The internal reference gene used was EVM0013809/*cdc-42*. The information of all primers was shown in Supplementary File S1. Each experiment was performed in triplicate for each biological replication.

3. Results

3.1. Atearic Acid Promotes Development of L. marina on CeMM

To determine whether stearic acid promotes development of *L. marina* on chemical defined food environment, we transferred newly hatched L1 larvae of *L. marina* to sea-salt-CeMM plates with stearic acid supplementation. We found that dietary stearic acid supplementation significantly promoted *L. marina* development on sea-salt-CeMM (Figure 1). Compared to the control (DMSO supplementation), the proportion of adults in the stearic acid supplementation group was significantly increased after 9 days post-hatching, and the adulthood rate at 15 days was significantly increased from about 40% (control) to nearly 80% (Figure 1). In line with the finding that stearic acid supplementation

promotes development of *C. elegans* on CeMM [23], our data showed that stearic acid was able to promote the development of marine nematode *L. marina* on sea-salt CeMM medium, indicating a conserved developmental regulation role of stearic acid.

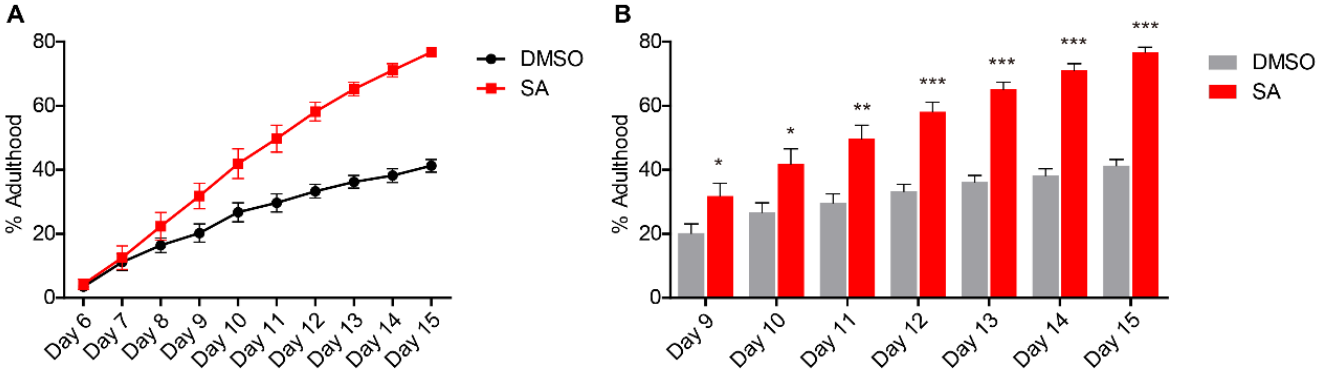

Figure 1. Stearic acid promoted *L. marina* growth on sea-salt-CeMM diet. (**A**,**B**) Daily adult rates of *L. marina* with dietary supplementation of SA (stearic acid) or DMSO (solvent control). (**A**) and (**B**) are different representations of the same set of data. DMSO, 6 trials, $n = 52$ worms on average in each trial; SA, 6 trials, $n = 52$ worms on average in each trial. All data shown are mean ± SEM (standard error of mean). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-sided *t*-test.

3.2. RNA-Seq Analysis in L. marina on Sea-Salt-CeMM with Stearic Acid Supplementation

To investigate transcriptomic responses of *L. marina* to dietary stearic acid supplementation, we performed high-throughput RNA-seq analysis. Newly hatched *L. marina* L1 larvae were cultured for 2.5 h on sea-salt-CeMM with stearic acid supplementation and DMSO (control) supplementation, respectively (Figure 2A). In total, 551 DEGs were identified, of which 289 were upregulated and 262 were repressed (Figure 2A,B). Details of significantly up- and downregulated DEGs are presented in Supplementary File S2.

KEGG and GO enrichment analysis results are shown, respectively in Figures 3 and 4 and Supplementary File S3: Figures S1 and S2.

3.3. Upregulation of Aminoacyl-tRNA Biosynthesis Pathway Genes

Based on KEGG analysis, we observed that the "aminoacyl-tRNA biosynthesis" pathway-related genes were significantly enriched in upregulated DEGs (Figure 3A). The expression levels of five amino-acyl tRNA synthetase genes such as EVM0000918/*wars-1* (tryptophanyl amino-acyl tRNA synthetase), EVM0003985/*cars-1* (cysteinyl amino-acyl tRNA synthetase), EVM0009296/*gars-1* (glycyl amino-acyl tRNA synthetase), EVM0009790/*nars-1* (asparaginyl amino-acyl tRNA synthetase) and EVM0011158/*kars-1* (lysyl amino-acyl tRNA synthetase) were significantly upregulated with dietary stearic acid supplementation (Figure 5A). Aminoacyl-tRNAs are essential substrates for protein translation [33]. Upregulation of aminoacyl-tRNA biosynthesis pathway genes might enable more proteins to be translated to support cell division and differentiation in development [33–36].

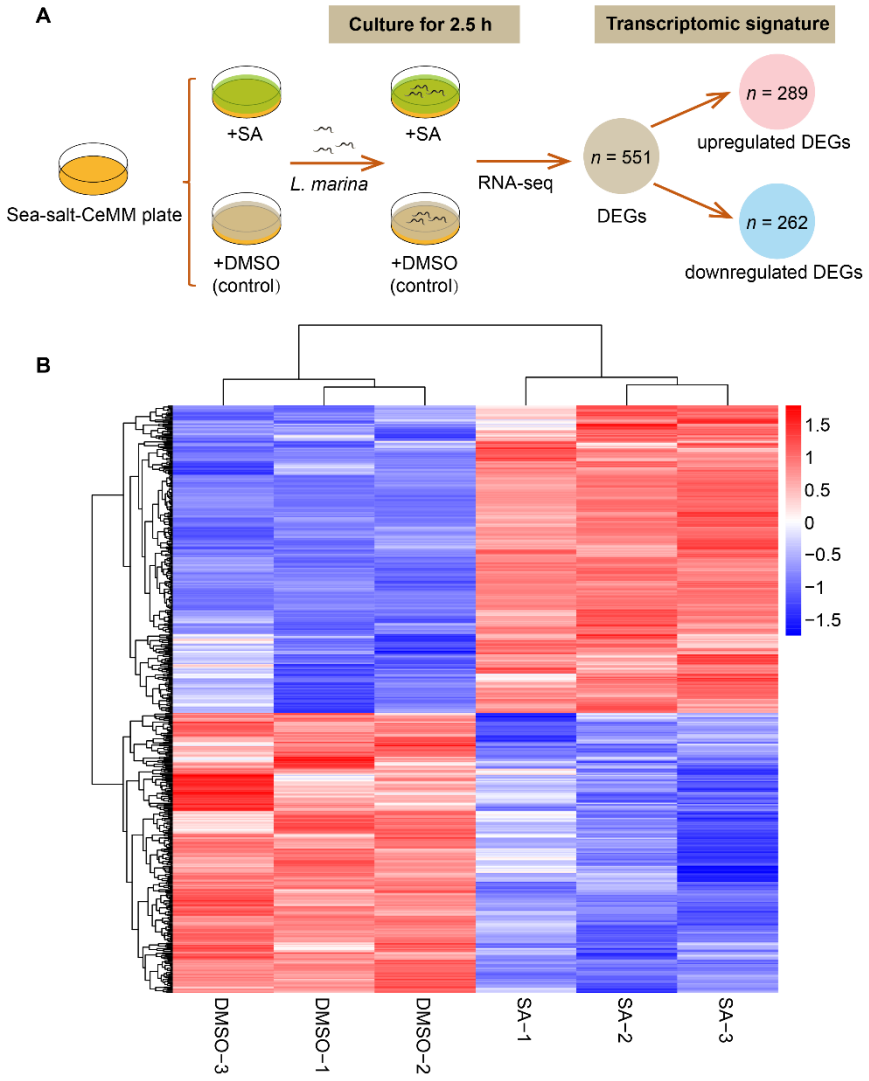

Figure 2. Transcriptome characterization of *L. marina* supplemented with dietary stearic acid (SA). (**A**) Experimental design and the transcriptomic characterization of dietary stearic acid supplementation. (**B**) Hierarchical clustering of DEGs (differentially expressed genes) in *L. marina* supplementation with stearic acid and DMSO using the pheatmap package in R. Relative expression levels (z-score, gene expression level based on base-2 log-transformed FPKM value) were indicated for each gene (row) in each sample (column). Red indicates upregulation; blue indicates downregulation. The scale bar shows the z-score for DEGs.

3.4. Upregulated Expression of Translation Initiation, Elongation, and Ribosome Biogenesis-Related Genes

We observed that "RNA transport" KEGG pathway genes were significantly upregulated in *L. marina* with dietary stearic acid supplementation (Figure 3A). RNA transport is a process in which RNA molecules are actively transported from one position within the cell to another, including the export of RNA from the nucleus to the cytoplasm through the nuclear pores, and the microtubule-assisted movement of specific RNAs along the axon [37–39]. Among eight upregulated DEGs enriched in the "RNA transport" pathway, five of which were translation initiation or elongation factors, including EVM0011266/*eif-1.A* (eukaryotic translation initiation factor 1A), EVM0015069/*eif-2Bbeta*, EVM0003319/*eif-2Bdelta*, EVM0016427/*eif-5*, and EVM0014509/*eef-1A.1* (eukaryotic translation elongation factor 1α) (Figure 5B).

Figure 3. KEGG enrichment analysis for DEGs. (**A**) The top 20 enriched KEGG pathways for upregulated DEGs. (**B**) The top 20 enriched KEGG pathways for downregulated DEGs. Significance of enrichment is indicated by color from red to purple. Gene ratio is the ratio of the number of DEGs annotated to the KEGG pathway to the total number of DEGs.

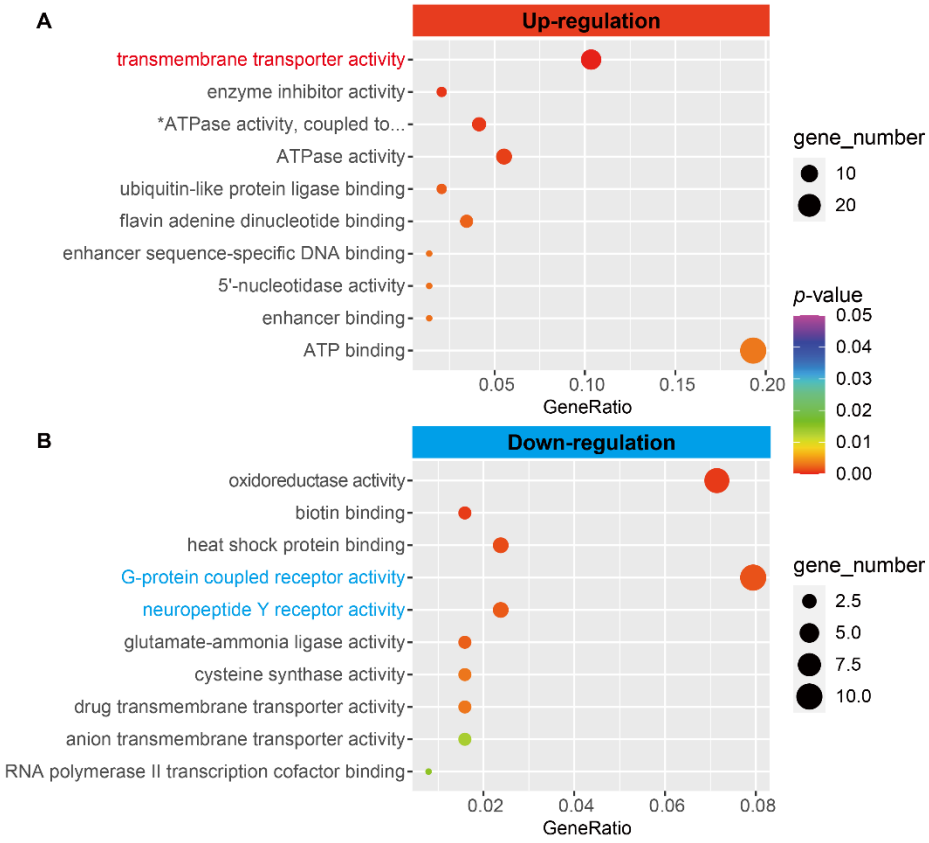

Figure 4. GO-MF (molecular function) enrichment analysis for DEGs. (**A**) The top 10 enriched GO-MF terms for upregulated DEGs. * The full name of this term is "ATPase activity, coupled to transmembrane movement of substances". (**B**) The top 10 enriched GO-MF terms for downregulated DEGs. Significance of enrichment is indicated by color from red to purple. Gene ratio is the ratio of the number of DEGs annotated to the GO term to the total number of DEGs.

In addition, we found that the expression of several genes in the "ribosome biogenesis in eukaryotes" pathway was significantly increased in *L. marina* with dietary stearic acid supplementation (Figure 3A). One of them was EVM0003025/*eif-6* (eukaryotic translation initiation factor 6), which is also a translation initiation factor. The other four were EVM0013635/*F27C1.6*, EVM0005281/*F55F10.1*, EVM0016791/*M01G5.3*, and EVM0007932/*ZK795.3* (Figure 5C). Ribosome biogenesis is the process of producing ribosomes, the macromolecular machines that are responsible for mRNA translation into proteins [40,41].

3.5. The Expression of Transmembrane Transporters Related Genes Was Upregulated

Based on GO functional enrichment analysis, we found that "transmembrane transporter activity" was the most significant enriched term for upregulated DEGs in MF (molecular function) category (Figure 4A). We observed that "transmembrane transporter activity"-related genes such as EVM0003002/*aat-5*, EVM0017539/*F14E5.1*, EVM0004825/*F23F1.6*, EVM0000321/*F41C3.2*, EVM0013480/*hmt-1*, EVM0007205/*mct-6*, EVM0004219/*mfsd-8*, EVM0011858/*pgp-9*, and EVM0003073/*slc-17.2* were significantly increased in *L. marina* with dietary stearic acid supplementation (Figure 5D).

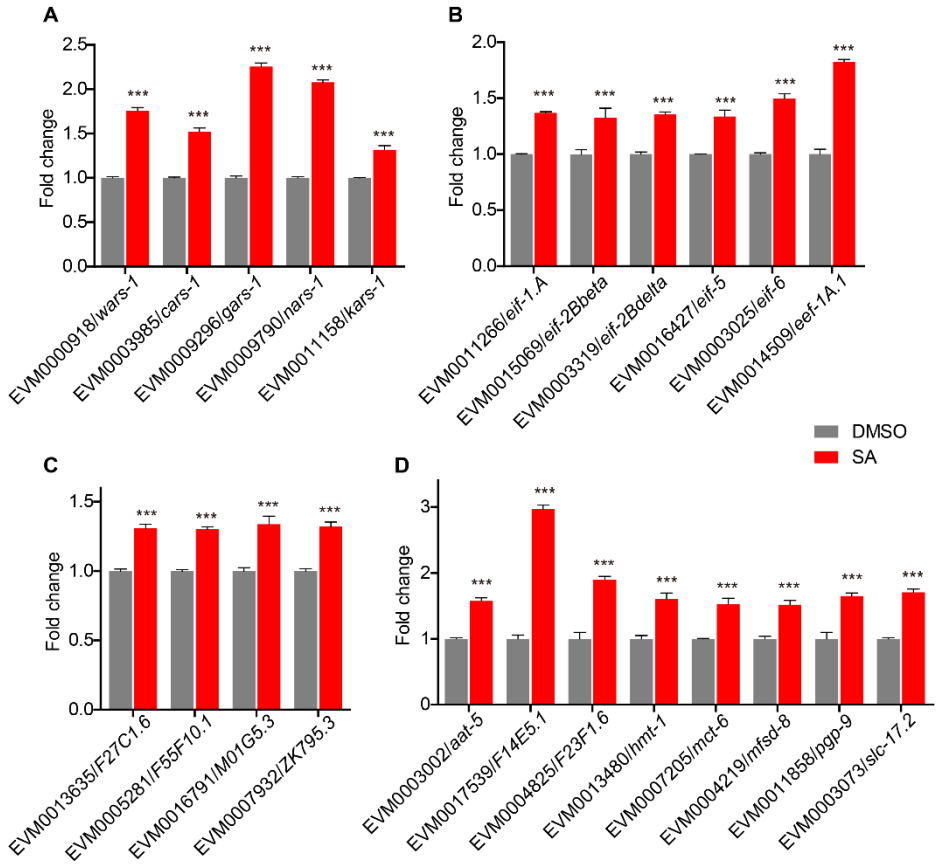

Figure 5. Expression of related upregulated pathway genes. (**A**) Expression level of aminoacyl-tRNA biosynthesis genes. (**B**) Expression level of translation initiation and elongation factors genes. (**C**) Expression level of ribosome biogenesis-related genes. The expression level of upregulated gene EVM0003025/*eif-6* enriched in this term is presented in (**B**). (**D**) Expression level of some transmembrane transporters-related genes. SA (stearic acid). The error bars represent SEM of the mean. *** *p*adj < 0.001.

3.6. Downregulation of Neuronal Signaling Genes

We found that genes related to KEGG pathway term "neuroactive ligand-receptor interaction" and GO terms "G-protein coupled receptor activity" and "neuropeptide Y receptor activity" were significantly decreased in *L. marina* with dietary stearic acid supplementation (Figures 3B and 4B). G protein-coupled receptors (GPCRs) are cell surface receptors that detect molecules outside the cell and could be activated by a variety of ligands such as hormones and neurotransmitters [42] and neuropeptide Y receptors belong to the class A GPCRs [43]. Significantly down-regulated "neuroactive ligand-receptor interaction" pathway genes were EVM0014526/*daf-37*, EVM0007244/*daf-38*, EVM0016479/*dop-4*, EVM0003946/*frpr-1*, EVM0006577/*frpr-3*, EVM0009311/*gnrr-5*, EVM0014635/*mod-1*, and EVM0011288/*ntr-1* (Figure 6A). Downregulated "G-protein coupled receptor activity" genes include EVM0007244/*daf-38*, EVM0016479/*dop-4*, EVM0010643/*F52D10.4*, EVM0003946/*frpr-1*, EVM0006577/*frpr-3*, EVM0015877/*frpr-7*, EVM0009311/*gnrr-5*, EVM0014635/*mod-1*, EVM0004184/*npr-5*m and EVM0011288/*ntr-1*, among them EVM0007244/*daf-38*, EVM0016479/*dop-4*, EVM0003946/*frpr-1*, EVM0006577/*frpr-3*, EVM0009311/*gnrr-5*, EVM0014635/*mod-1*, and EVM0011288/*ntr-1* were also enriched in "neuroactive ligand-receptor interaction" KEGG pathway (Figure 6B). Moreover, we observed that "neuropeptide Y receptor activity" term genes such as EVM0009621/*npr-7*, EVM0000777/*npr-10*, and EVM0007986/*npr-13* were significantly repressed in *L. marina* with dietary stearic acid supplementation (Figure 6C).

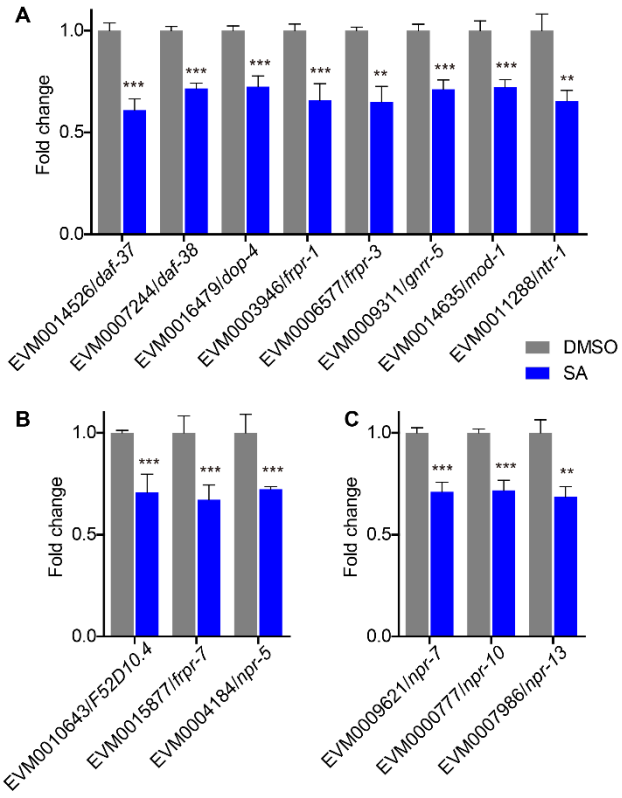

Figure 6. Downregulated expression of neuronal signaling related genes. (**A**) Expression level of "neuroactive ligand-receptor interaction" downregulated genes. (**B**) Expression level of "G-protein coupled receptor activity" genes. The expression level of downregulated genes EVM0007244/*daf-38*, EVM0016479/*dop-4*, EVM0003946/*frpr-1*, EVM0006577/*frpr-3*, EVM0009311/*gnrr-5*, EVM0014635/*mod-1*, and EVM0011288/*ntr-1* enriched in this term is presented in (**A**). (**C**) Expression level of "neuropeptide Y receptor activity" genes. SA (stearic acid). The error bars represent SEM of the mean. ** *p*adj < 0.01, *** *p*adj < 0.001.

3.7. Quantitative Real-Time PCR Validation

We conducted qPCR to test the expression of interest genes identified from our RNA-seq results (Figure 7A). The qPCR results and RNA-seq results showed consistent trends (Figure 7B, Supplementary File S4). The expression of aminoacyl-tRNA biosynthesis genes EVM0009296/*gars-1* and EVM0009790/*nars-1*, translation initiation factor gene EVM0003025/*eif-6*, ribosome biogenesis-related genes EVM0016791/*M01G5.3* and EVM0007932/*ZK795.3*, and transmembrane transport-related genes EVM0017539/*F14E5.1* and EVM0007205/*mct-6* was significantly upregulated in *L. marina* with stearic acid supplementation, while the expression of neuronal signaling-related genes such as EVM0014526/*daf-37*, EVM0003946/*frpr-1*, and EVM0009311/*gnrr-5* was significantly downregulated in *L. marina* with stearic acid supplementation.

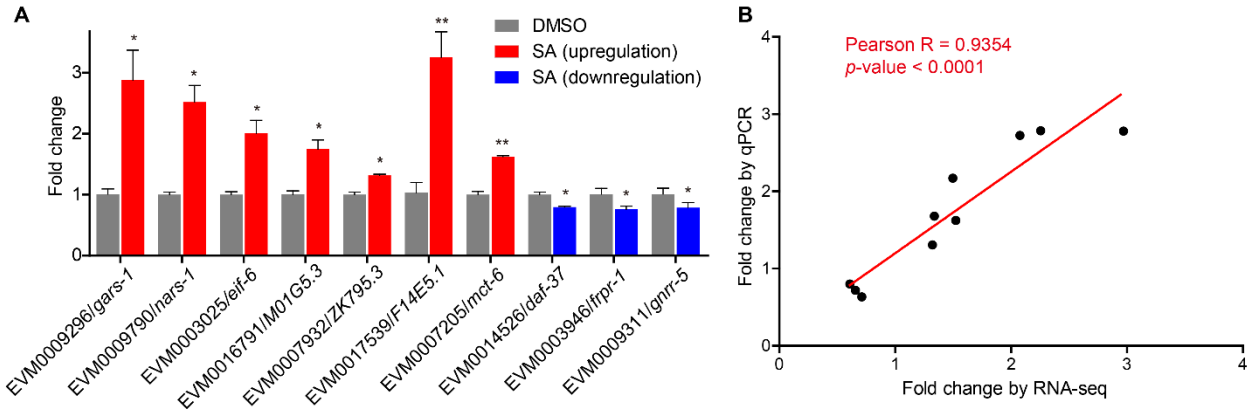

Figure 7. Validation of the RNA-seq results using qPCR. (**A**) qPCR analysis of interest genes identified from the RNA-seq results. Fold changes indicate the ratio of the SA (stearic acid) supplementation group to the control DMSO group. The error bars represent SEM of the mean, * $p < 0.05$, ** $p < 0.01$, two-sided *t*-test. (**B**) Correlation analysis of the results of RNA-seq and qPCR for detected genes. Each dot represents a gene, with detailed information shown in Supplementary File S4. Correlation analysis was performed using GraphPad Prism 5 software, Pearson R = 0.9354, with a *p*-value < 0.0001.

4. Discussion

It was reported that stearic acid suppresses the development of the mammary gland in mice [44] and inhibits tumor development in rats [45]. A previous report showed that dietary stearic acid interferes with polyunsaturated fatty acid required for tumor development in mice models [46]. In addition, stearic acid inhibits the growth of human cervical cancer cells [47]. Another study showed that mice fed a diet containing stearic acid exhibit increased food intake and hedonic eating [48]. In *Drosophila melanogaster*, stearic acid supplementation improves animal physiology and mitochondrial function [49]. Certain concentrations of stearic acid can prolong the lifespan of *C. elegans*, while excessive stearic acid shortens the body length and longevity [50]. In this report, we found that dietary stearic acid supplementation promoted development of *L. marina* on sea-salt-CeMM (Figure 1), in line with our previous report that stearic acid supplementation promotes the growth of *C. elegans* on CeMM [23].

4.1. Stearic Acid Might Promote L. marina Development through Upregulation of Protein Synthesis Related Genes

Aminoacyl-tRNA synthetases (ARSs) ligate amino acids to the corresponding tRNAs in protein synthesis [36,51], and has been a growth marker for multiple zooplankton because of its positive correlation with individual growth rate [34,35]. Aminoacyl-tRNA synthetase defects lead to protein mistranslation and affect cellular physiology and development [52]. In cultured human HeLa cells, the disruption of MARS (multi-aminoacyl-tRNA synthetase complex) results in growth retardation due to a decreased rate of protein synthesis [53,54]. Drugs targeting methionyl-tRNA synthetases inhibit protein synthesis and consequently inhibit malaria parasite development [51]. In *C. elegans*, mutant of the *glp-4* gene encoding valyl aminoacyl tRNA synthetase has reduced protein translation, displays slow growth, and shows defects in germline development [55]. A quantitative proteome analysis of *C. elegans* found that the level of valyl aminoacyl tRNA synthetase (encoded by *glp-4*) and aspartyl aminoacyl tRNA synthetase (encoded by *dars-1*) is significantly increased at 40 h after hatching compared to 20 h post hatching, suggesting that the increased aminoacyl-tRNA synthetases are critical for nematode development [56]. In the present study, we found that *L. marina* aminoacyl-tRNA synthetases genes, such as EVM0000918/*wars-1*, EVM0003985/*cars-1*, EVM0009296/*gars-1*, EVM0009790/*nars-1*, and EVM0011158/*kars-1*, were significantly upregulated with dietary stearic acid supplementation (Figure 5A). Our

data indicate that stearic acid supplementation might promote development of *L. marina* via upregulating aminoacyl-tRNA synthetases to induce protein synthesis.

Eukaryotic initiation factors (eIFs) are essential in the initiation stage of eukaryotic translation, and contribute to the stabilization of ribosomal preinitiation complexes formation around the start codon [57,58]. Mutation of the eIF-5A homologue *iff-2* or partial reduction of the expression of eIF-5B coding gene *iffb-1* delays larval development in *C. elegans* [59,60]. Translation elongation factors help to elongate the polypeptide chain in translation [61]. A study in yeast has shown that elongation factor 1β mutant reduced total protein synthesis and growth defects [62]. *C. elegans* translation elongation factor 4 (EF4) localizes to mitochondria, and EF4 impairment delays development [63]. These reports suggest that translation initiation and elongation factors critically mediate the protein synthesis process and their deficiency might inhibit animal growth. In the present study, we found that several translation initiation and elongation factors such as EVM0011266/*eif-1.A*, EVM0015069/*eif-2Bbeta*, EVM0003319/*eif-2Bdelta*, EVM0016427/*eif-5*, and EVM0014509/*eef-1A.1* were significantly upregulated in *L. marina* with dietary stearic acid supplementation (Figure 5B). Among them, previous studies have reported that RNAi inhibition of *eif-1.A* [64,65], *eif-2Bbeta* [66], *eif-2Bdelta* [64], or *eif-6* [66] all slow down the growth of *C. elegans*. Therefore, our data indicate that stearic acid supplementation might promote development of *L. marina* on CeMM through inducing the expression of translation initiation and elongation factors genes.

Ribosomes are the fundamental macromolecular organelles that play a central role in the translation process, allowing the information encoded within mRNA to be converted into proteins [40]; a large number of ribosomes are required for cell growth [41]. A previous report showed that RBD-1 is essential for the early development of *C. elegans* through 18S ribosomal RNA processing [67]. The *C. elegans* nucleostemin promotes cell growth via increasing the ribosome biogenesis [68]. Here, we found that the expression of several ribosome biogenesis-related genes was significantly upregulated in *L. marina* with dietary stearic acid supplementation (Figure 5C), indicating that stearic acid supplementation might promote *L. marina* development via upregulation of ribosome biogenesis-related genes.

4.2. Stearic Acid Might Promote L. marina Development via Upregulation of Transmembrane Transporter Genes

Transmembrane transporters facilitate nutrient sensing and transport [69–71], and have been implicated in various diseases [72–74]. In this study, we observed that the expression of multiple transmembrane transporter genes such as *aat-5*, *hmt-1*, *mct-6*, *mfsd-8*, *pgp-9*, and *slc-17.2* were significantly increased in marine nematode *L. marina* supplemented with stearic acid (Figure 5D). *aat-5* encodes a L-amino acid transmembrane transporter, *hmt-1* enables cadmium ion transmembrane transporter activity, *mct-6* encodes a monocarboxylic acid transmembrane transporter, *mfsd-8* is an ortholog of human MFSD8 and enables transmembrane transporter activity, *pgp-9* is an ortholog of human ABCB1 and ABCB4, and *slc-17.2* is predicted to be involved in anion transport (www.wormbase.org, accessed on 4 February 2022).

It was reported that RNAi knockdown of *aat-5* could partially rescue the reduced brood size in *C. elegans pept-1* (intestinal peptide transporter) mutant [75]. *C. elegans hmt-1*, a member of ABCB belonging to the ABC transporter superfamily, detoxifies cadmium, copper, and arsenic [76,77]. *C. elegans* ABCE is a transmembrane transporter, and RNAi knock-down the expression of *abce-1* leads to slow growth [78]. MFSD (major facilitator superfamily domain) family members are known to be involved in energy consumption and homeostasis [79–82]. A previous report showed that *Mfsd2a* knockout mice are smaller and leaner [80], and *mfsd-6* promotes PLM (posterior lateral microtubule) regrowth in *C. elegans* [83]. *gem-1*, encoding the SLC16 monocarboxylate transporter-related protein, promotes gonadal cell divisions in *C. elegans* [84]. *T09F3.2* encodes a pyrimidine nucleotide transporter belonging to the SLC transporter family, and *C. elegans T09F3.2* mutant exhibits slow growth and movement [85]. Together, our data suggest that stearic acid supplementa-

tion might promote development of *L. marina* via upregulation of certain transmembrane transporter genes.

4.3. Stearic Acid Might Promote Development of L. marina through Reduced Neuronal Signaling

Neuronal signaling is essential for nutrition sensation, feeding behavior, and developmental regulation in animals [16,86–88]. In particular, acetylcholine neuronal signaling plays an essential role in regulating diet consumption and energy homeostasis [89,90]. Impairment of cholinergic signaling increases food intake and leads to obesity [90]. Nicotine, as a known appetite suppressant, was reported to reduce food intake by activating nicotinic acetylcholine receptors (nAChRs) [91]. We previously reported that transmembrane channel-like gene *tmc-1* attenuates the development of *C. elegans* via nicotinic acetylcholine receptor gene *eat-2* [16], and this developmental regulating effect of *tmc-1*/*eat-2* is achieved by altering fatty acid metabolism [23]. Our transcriptomic data showed that dietary stearic acid supplementation did not significantly change the expression of *tmc-1* and *eat-2* genes in *L. marina*, while we identified more than a dozen neuronal signaling receptor genes such as *daf-37*, *daf-38*, *dop-4*, *frpr-1*, *frpr-3*, *frpr-7*, *gnrr-5*, *mod-1*, *npr-5*, *npr-7*, *npr-10*, *npr-13*, and *ntr-1* that were significantly downregulated in *L. marina* with dietary stearic acid supplementation (Figure 6). Both *daf-37* and *daf-38* encode G protein-coupled receptors, which cooperatively mediate pheromone perception and are involved in dauer formation in *C. elegans* [92]. DOP-4 protein is a dopamine neurotransmitter receptor [93], *frpr-1*, *frpr-3*, and *frpr-7* all belong to the FMRFamide peptide receptor gene family, *gnrr-5* is a homolog of human gonadotropin-releasing hormone receptor (GnRHR) gene, *mod-1* is a serotonergic chloride channel, *npr-5*, *npr-7*, *npr-10*, and *npr-13* belong to the neuropeptide Y receptor family, and *ntr-1* is a homolog of human AVPR1B and AVPR2 (www.wormbase.org, accessed on 4 February 2022).

A previous report showed that the *dop-4* mutants are shorter compared to the wild-type *C. elegans* [94]. External food supply promotes *C. elegans* avoidance responses to soluble repellents in a *dop-4* dependent manner [95]. *C. elegans* FRPR-3 serves as a receptor for the FLP-20 (FMRF-like peptide 20) released by primary mechanosensory neurons, and regulates arousal and other behavioral states [96]. *C. elegans* AVK interneurons mediate food sensation by releasing the FLP-1 neuropeptides and the receptors are NPR-6 and FRPR-7 [97]. *mod-1* is required for locomotor rate reduction when food-deprived *C. elegans* re-encounter food [98], and is involved in serotonin-induced fat loss [99]. NPR-1 is reported to regulate *C. elegans* foraging [100], and a previous report showed that *npr-5* encodes a receptor gene for the FLP-18 neuropeptide, and *flp-18* mutants exhibits defects in chemosensation and foraging [101]. It has been reported that FMRFamide neuropeptides inducing *C. elegans* sleep act via NPR-7 [102]. NPR-10 encodes a receptor for neuropeptides encoded by *nlp-14*, and NLP-14 activates NPR-10 to repress serotonin-induced aversive responses [103]. NTR-1 is a receptor for nematocin, which is a vasopressin/oxytocin-related neuropeptide. Previous reports showed that nematocin controls male mating behaviors, as well as salt chemotaxis of gustatory associative learning in *C. elegans* [104,105]. Given that impairing the function of acetylcholine neuronal signaling genes such as *tmc-1* or *eat-2* promote *C. elegans* development on CeMM [16,23], our data indicate that stearic acid supplementation might accelerate the growth of *L. marina* via repressing the expression of dopamine, serotonin, FMRFamide peptide, neuropeptide Y, and other neural receptor genes.

5. Conclusions

In conclusion, we found that dietary stearic acid supplementation promotes development of the marine nematode *L. marina* on an axenic chemical defined sea-salt-CeMM medium, indicating a conserved role of stearic acid in developmental regulation. Our results provide important insights into how a single fatty acid stearic acid regulates animal development, together with the advantage that the composition and amount of each chemical can be manipulated in the chemically defined CeMM medium. Further research with genome editing might illustrate the molecular mechanisms underlying how a single

metabolite regulates animal development and physiology in response to global climate change with a dynamic nutritional environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse10030428/s1, Supplementary File S1: Information of qPCR primers. Supplementary File S2: Details of differentially expressed genes. Supplementary File S3: Supplementary figures, Figure S1: GO-BP (Biological Process) enrichment analysis for DEGs; Figure S2: GO-CC (Cellular Component) enrichment analysis for DEGs. Supplementary File S4: Correlation analysis of the results of RNA-seq and qPCR for detected genes.

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