The effects of 5 aminolevulinic acid supplementation in Pacific white shrimp Litopenaeus vannamei

[課程博士・論文博士共通]

Doctoral Dissertation Summary

専　攻 Major	APPLIED MARINE BIOSCIENCES	氏　名 Name	ヘラスミオ イバネ ペドロサ GERASMIO IVANE PEDROSA
論文題目 Title	The effects of 5-aminolevulinic acid supplementation in Pacific white shrimp *Litopenaeus vannamei* (バナメイエビ *Litopenaeus vannamei* における 5-アミノレブリン酸の経口投与がもたらす効果)		

Dissertation research topics:

I. Effects of 5-aminolevulinic acid on gene expression, immunity, and ATP levels in Pacific white shrimp, *Litopenaeus vannamei*
   (This part was published in Marine Biotechnology (2018) 20(6), 829-843 with doi:10.1007/s10126-018-9852-2; Publisher: Springer Verlag)
II. Dietary 5-aminolevulinic acid enhances adenosine triphosphate production, ecdysis and immune response in Pacific white shrimp, *Litopenaeus vannamei* (Boone)
   (This part was published in Aquaculture Research (2019) 50(4), 1131-1141 with doi:10.1111/are.13987; Publisher: John Wiley & Sons Ltd).)
III. Bacterial community profile of *Litopenaeus vannamei* after 5-aminolevulinic acid supplementation (Submitted to the Journal of Applied Microbiology with the title "5-Aminolevulinic acid supplementation promotes beneficial bacterial community structure in Pacific white shrimp *Litopenaeus vannamei*")
IV. Molecular cloning, characterization and gene expression analysis of 5-aminolevulinic acid synthase gene in *Litopenaeus vannamei* (Submitted with the same title to the journal Gene)

Purpose:

The general purpose of this dissertation is to evaluate the effects of 5-aminolevulinic acid (5-ALA; a precursor of heme) supplementation in Pacific white shrimp *L. vannamei*. I also determined 5-ALA's potential as an alternative to antibiotics in controlling acute hepatopancreatic necrosis diseases (AHPND), an emerging disease in shrimp aquaculture caused by a toxin-producing strain of *Vibrio parahaemolyticus*. Specific purposes for each research topic are the following:

I. To examine the effects of 5-ALA on the gene expression, immunity and ATP levels of Pacific white shrimp *L. vannamei.*
II. To investigate the effects of 5-ALA on growth and several health-related characteristics of *L. vannamei* including ATP level, total hemocyte count, immune response to *V. parahaemolyticus* infection and the expression of ecdysis- and immune-related genes (including some hemoproteins).
III. To determine the effect of 5-ALA supplementation (15 ppm) on the bacterial community profile of *L. vannamei.*
IV. To better understand ALA synthase in *L. vannamei* (LvALAS), cloning, characterization, gene expression analysis, and gene silencing of LvALAS were conducted.

Methodology/Experiments/Analysis:

I. Feeding experiment (15-ppm 5-ALA supplementation vs. control group fed with basal diet), gene expression profile by microarray analysis, validation of microarray analysis results by quantitative PCR (qPCR), gene expression of some hemoproteins and genes associated with heme synthesis and degradation, immersion challenge test and ATP level assay.

II. Feeding experiment (three 5-ALA concentrations were used and compared with the control group after 12 weeks of feeding). Dietary groups included the control group fed with BD and 5-ALA groups fed with BD plus 15, 30 and 60 ppm of 5-ALA (ALA15, ALA30 and ALA60, respectively). Since it was previously mentioned that 5-ALA upregulated genes involved in chitin binding and metabolism, an investigation on the effect of 5-ALA on ecdysis (=molting) was added. ATP level assay, growth parameters and molting frequency, expression of ecdysis-related and immune-related genes after 12 weeks of feeding, expression of immune-related genes after bacterial infection, total hemocyte count, and immersion challenge test were performed/investigated.

III. Stomach and intestinal bacterial structure and function after 5-ALA supplementation were determined by analyzing the V4 region of the 16S rRNA gene using MiSeq Illumina sequencing technology. Pre-processing of reads and data analyses were done using R and RStudio. The R script used in this study can be found at Github (https://github.com/ivanepg/16S-microbiome-analysis-in-R).

IV. Cloning of the full open reading frame of LvALAS, LvALAS sequence analysis, gene expression analysis of LvALAS splice variants, LvALAS tissue distribution, age-dependent gene expressions of LvALAS, Cytochrome oxidase subunit I (COX I) and subunit IV (COX IV), gene expression of LvALAS and catalase after *V. parahaemolyticus* infection, LvALAS gene silencing were performed/investigated.

Results:

I. Of the 15,745 *L. vannamei* putative hepatopancreas genes spotted on the microarray, 101 were DEGs, i.e., transcript levels differed by more than fourfold, between the 5-ALAsupplemented and control groups at $p< 0.05$. Based on sequence homology of known genes, 41 (41/101) genes were immune- and defense-related. Interestingly, seven of these genes are involved in chitin binding and metabolism and 5 of these 7 genes were highly upregulated, by more than eight-fold in 5-ALA group compared to the control. 5-ALA-supplemented shrimp also had a higher ATP level and survival rate in the challenge test (95%) compared to the control (0%) two weeks post infection. The heme synthesis pathway genes (porphobilinogen synthase and ferrochelatase) and hemoproteins (catalase and nuclear receptor E75) were also relatively higher in 5-ALA group compared to the control group.

II. After 12 weeks of feeding, most 5-ALA diets increased the expression of genes associated with ecdysis (nuclear receptor E75, cytochrome P450 Shade and chitinases 3 and 4) compared to the control. Ecdysis frequency and growth were highest in ALA30, although growth difference was not statistically significant compared to the control group. CAT and proPO gene expression levels were also significantly higher in ALA60 after the 12-week feeding trial. Six hours post-*V. parahaemolyticus* infection, THC (ALA60) and CAT gene expression levels were significantly higher in 5-ALA groups (ALA30, ALA60) than the control. ALA15 and ALA60 diets also increased resistance of *L. vannamei* against *V. parahaemolyticus*, with higher survival rate (90%) compared to the control (50%). The findings of higher mRNA expressions of some immune-related genes in the hepatopancreas, higher THCs and higher challenge test survival rates in the 5-ALA groups support the hypothesis that administration of 5-ALA enhances immune response in *L. vannamei*. Also, the findings that 5-ALA enhances the expression of some ecdysis-related genes (e.g., the hemoproteins E75 and Shade) and increases the number of molts suggest that 5-ALA also induces ecdysis.

III. Differentially abundant taxa having potential benefits like *Enhydrobacter* and *Oceaniovalibus* were higher in 5-ALA group while potential pathogens like *Tenacibaculum* and *Mycobacterium* were

more abundant in the control group. For the microbiome function prediction, control group have higher counts matching with the KEGG pathway 'human diseases' both in the intestine and stomach, compared to 5-ALA group, after 1 week of supplementation.

IV. Cloning revealed that LvALAS is composed of an open reading frame of 1599 bp, encoding 532 aa and is ubiquitously present in all the *L. vannamei* tissues investigated. LvALAS gene expression levels (a) significantly decreased with age in the intestine and hepatopancreas; (b) significantly increased in the stomach and slightly increased in other tissues after bacterial infection; and, (c) decreased after 12- and 24- post injection in LvALAS-silenced shrimp, compared to the PBS-injected group. It was also observed that LvALAS-silenced shrimp cannot not molt normally even if they were already at the late pre-molt stage (compared to the PBS-injected control group which molted every 8-10 days) and died eventually after 2 weeks post injection.

Conclusion:

I. The results of this study demonstrate that dietary administration of 5-ALA upregulates various immune- and defense-related genes (including some hemoproteins), increases ATP level and improves resistance to AHPND-causing *V. parahaemolyticus*. Further studies are needed to determine its effect on molting and growth as well as its effects when used for a longer period and in different concentrations.

II. The results showed that dietary administration of 5-ALA increases ATP levels, enhances the immune response and induces ecdysis in *L. vannamei.* Of the three 5-ALA groups, ALA60 had the strongest immune response but at the same time, the lowest growth performance. Thus, further studies are needed on the optimal feeding schedule and concentration of 5-ALA. In addition, further studies on the effect of 5-ALA on ecdysis appear promising because of the important effect molting has on growth and reproduction in shrimp.

III. Taken together, the results show that 5-ALA supplementation modified the bacterial structure in the intestine and stomach of *L. vannamei*, and this alteration may promote the formation of a beneficial bacterial community in shrimp. These results may also help to explain recent findings that 5-ALA promotes the health status of shrimp.

IV. The findings of this study show that *L. vannamei*, like other invertebrates, has only the housekeeping form of ALAS. LvALAS mRNA transcripts decreased with age and increased following bacterial infection. LvALAS dsRNA-injected shrimp could not molt, suggesting that an ALAS-dependent pathway is necessary for molting.