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FULL PAPER

Research on the *Artemia* nauplii microorganism compositions based on metagenomics next-generation sequencing

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SUMMARY

Vibrio species represent the predominant and significant pathogen in global marine fish and shellfish aquaculture. Vibrio species are ubiquitously presented in Artemia cyst hatcheries, and their notable colonization in live prey, particularly Artemia nauplii, leads to the transmission of these pathogens into the digestive system of larval organisms, causing serious problems in Vibriosis in marine aquaculture. To eliminate the *Vibrio*species in *Artemia* nauplii, trichloroisocyanuric acid (TCCA) was used for sterilization of the nauplii. In this study, 3 different concentrations, including 0.5 ppm (FA group), 1.0 ppm (FB group) and 1.5 ppm (FC group) of TCCA were used to treat nauplii for 25 min, and then genomic DNA of the different groups were extracted followed by metagenomic next-generation sequencing (NGS). Bioinformatics analysis was applied and the results indicated that Proteobacter constituted the predominant component within each group at the phylum level, albeit accounting for only 58.68% in the FB group, which was significantly lower than in other groups (>86%). The relative abundance of *Vibrio* species at genus level showed that when compared with the control group, the FB group (15.8%) was reduced by 25.5%. Beta diversity showed differences between the FB group and the other groups, suggesting that treatment with 1.0ppm TCCA for 25 min would obviously reduce the Vibrio in *Artemia* nauplii. In conclusion, the *Vibrio* species were significantly reduced after treatment with TCCA, indicating that TCCA might be an alternative to antibiotics used for live food sterilization in marine aquaculture.

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INTRODUCTION

*Artemia salina (*A. salina*)* belonging to Crustacea, is a primitive aquatic arthropod of the *Artemiidae* family (Zhang *et al.*, 2021). Their remarkable resistance to changes ensures that they can live in a wide range of seawater systems, from swamps to lakes. It is believed that Artemia can tolerate a salt concentration of up to 50%(De Vos *et al.*, 2013). After hatching, Artemia salina reaches sexual maturity within 18-21 days and reproduces within the next two days. Females can switch from one mode of reproduction to the other in between two reproduction cycles. The adult female produces free-swimming larvae under optimal conditions, but in extreme conditions, cysts (oviparity) are produced, leading to diapause (Azra *et al.*, 2022; Gajardo *et al.*, 2012). Embryos have special tolerances for anoxia, ionizing radiation and extreme pressure. Just like plant seeds, the cysts stay alive for years and can be hatched under specific environmental stimulations (Chen *et al.*, 2021; Thorel *et al.*, 2020).

The nutritional value of Artemia salina makes it the most popular live food for marine aquarium fishes (Pecoraro *et al.*, 2021). Their low cost and ease of use make it ideal for feeding coral, larval fish and other crustaceans. In *Macrobrachium rosenbergii* culture, supplementation of live larvae helped improve feeding efficiency (Candelier *et al.*, 2019). Traditionally, *Artemia salina* nauplii were directly fed to shrimp without any further treatment. However, lots of microorganisms were propagated along with the *Artemia salina* larvae during the hatching process. The main microorganisms in nauplii including the Vibrio cholerae, Vibrio dazotrophicus, Alteromonas mediterranea and Marinomonas sp. Though most of the microorganisms are harmless to *Artemia salina* nauplii, their existence might cause disease in *Macro-*

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brachium rosenbergii (Valverde *et al.*, 2019). As we know, the *Vibrio* and *Vibrio*-related bacteria are important pathogens in shrimp larvae, as infection with them might lead to acute hepatopancreatic necrosis disease (AHPND) (Restrepo *et al.*, 2021). When feeding on nauplii with large scale of *Vibrio*, the increasing disease outbreaks in aquaculture would cause huge economic harm to the shrimp farming industry (Sanches-Fernandes *et al.*, 2022). Antibiotics had been widely used to prevent and control the disease in shrimp farming, which further intensified antibiotic resistance, inhibiting beneficial microorganisms and causing harmful effects in the development of aquatic animals (Defoirdt *et al.*, 2011; Schar *et al.*, 2021). Therefore, antibiotics were not a good solution to the *Vibrio* problem. A safer way was the reduction of pathogenic bacteria in *Artemia* nauplii; usually the cysts were run through the washing step before hatching into nauplii. However, it could not thoroughly eliminate the harmful bacteria in the live food fed to the shrimp larvae. A special method, such as sterilization of nauplii before feeding, was considered a substitute for antibiotics.

Trichloroisocyanuric acid (TCCA) is an efficient and versatile reagent for chlorination and oxidation. Trichloroisocyanuric acid, with highly solubility and low toxicity, was widely applied as a disinfectant and bleaching agent. Water treated with TCCA released cyanuric acid and hypochlorous acid, which acted against pathogenic microbes to sterilize water and preserve freshwater for swimming. In this study, TCCA was used for nauplii sterilization. To further evaluate the practicability of sterilized nauplii, microorganism compositions should be analyzed.

In the last few decades, with the development of next generation sequencing technologies, gene sequencing is becoming a more accessible process. In fact, metagenomic next generation sequencing (NGS) has been used to help disease diagnosis (Ankala *et al.*, 2015). Next-generation sequencing, also called deep sequencing or high-throughput sequencing, is a method of sequencing deoxyribonucleic acids (Cao *et al.*, 2017; Pervez *et al.*, 2022). The process of NGS involves five main steps: the collection of samples, nucleic acid extraction, database construction, sequencing, and data analysis (Malla *et al.*, 2018).

With the help of metagenomic tools, all the nucleic acid from the host to pathogenic specimens can be sequenced. This process is untargeted and able to identify pathogens regardless of their species, providing sufficient information about pathogens, including their antimicrobial genes and host responses (Hilt *et al.*, 2022; Malla *et al.*, 2018). Therefore, NGS has been widely used to analyze microorganisms (Fourgeaud *et al.*, 2023; Li *et al.*, 2022; Tarnecki *et al.*, 2018).

To find a suitable method for *Artemia* nauplii sterilization and further improve the biosafety of live food for shrimp seeding, in this study the *Artemia* nauplii were treated with three different concentrations of trichloroisocyanuric acid (TCCA) and further applied to metagenomic next-generation sequencing to determine the microorganism composition difference among different groups, which might help improve the quality of *Artemia salina* nauplii and further reduce the risk of disease outbreaks during *Macrobrachium rosenbergii* culturing.

MATERIAL AND METHODS

Hatching of Artemia cysts

Artemina cysts (Great Salt Lake strain, INVE, Thailand) preserved at 4°C were hatched by following the standard procedure (Liu *et al.*, 1987). In brief, the cysts were cleaned and sterilized before being spread on paper to dry the water overnight. The cyst membrane was removed by dissolution in sodium hypochlorite, while keeping the function of the embryo. Approximately 3 g of precleaned cysts were incubated in 1 L of 4% sodium hypochlorite solution in the conical hatching device. The incubation temperature was 27°C, and a small line running from an aquarium air pump maintained aeration at the bottom of the device. In addition, under these circumstances, Artemia hatched within 18 hours after exposure to light.

Sterilization of Artemia nauplii

The continuous aeration was ceased 10 min before the separation of *Artemia* nauplii. After the cysts hatched, the *Artemia* nauplii were collected with a150-μm plankton net and subsequently transferred into a 2-L fiberglass culture tank for further use. Then the nauplii were soaked in different concentrations of richloroisocyanuric acid (TCCA) for sterilization. The concentrations of TCCA were 0.5 ppm (FA group), 1 ppm (FB group) and 1.5 ppm (FC group), and all the nauplii were soaked for 25 min before being washed several times with fresh water. Nauplii that were not sterilized were used as a control group; in fact, two control groups, named AN and NA1. The difference between the two control groups was that the NA1 group was washed several times with sterilization water before genomic DNA extraction, while the AN group was run through DNA extraction directly after hatching without any washing step. All the groups were performed in triplicate.

Sample collection and DNA extraction

Three different TCCA concentration treated groups and two control groups of nauplii were collected and stored at -80°C before further use. DNA extraction was performed with the QIAamp Fast DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Negative extraction controls (DNA-free water) were run in the same way as samples. The DNA samples were tested by NanoDrop 2000 and agarose gel electrophoresis for concentration determinant

and quality control and further diluted with sterile water to 1 ng/μl.

PCR amplification

Amplicon libraries targeting 16SV4 region for bacteria, 18S V4 region for eukaryotic microbes and ITS1 region for fungi were prepared. All the primers were selected as recommended in the 16S Metagenomic Sequencing Library Preparation guide (http://emea.support.illumina.com). The 16S V4 primers were 515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3', the 18S primers were 528F: 5'-GCGGTAATTCCAGCTCCAA-3' and 706R: 5'-AATCCRAGAATTTCACCTCT-3' while the ITS1 primers were ITS5-1737F: 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS2-2043R: 5'-GCTGCGTTCTTCATCGATGC-3'. PCR amplification was carried out using the Phusion® High-Fidelity PCR Master Mix (New England Biolabs) at the following conditions: 1min at 98°C for predenaturation, followed by 30 cycles of amplification (98°C for 10s, 50°C for 30s and 72°C for 30s) and 5min at 72°C for final elongation before cooling to 4°C. All the samples were run in triplicates and the amplification products were purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as described in the 16S Metagenomic Sequencing Library Preparation guide (Logares *et al.*, 2014).

DNA library construction and metagenomic sequencing

Library preparation was performed with the TruSeq® DNA PCR-Free Sample Preparation Kit following the manufacturer's instructions (Van Nieuwerburgh *et al.*, 2011). To be brief, qualified DNA samples were sheared into small fragments, then blunt-end fragments were created. After ends reparation, fragments were narrowly size selected with sample purification beads. Then the Agilent 2100 Bionaylzer and ABI StepOnePlus Real-Time PCR system were used to qualify and quantify the sample libraries. The qualified libraries were sequenced via NovaSeq6000 platform (Novogene, Beijing, China) and 250bp paired-end reads were generated.

Taxonomic Classification

Sequencing reads from the platform were converted into the universally accepted format and both sequence trimming and QC analysis were performed for validation. The remaining sequencing data was aligned by BWA (Burrows-Wheeler alignment, http://bio-bwa.sourceforge.net) simultaneously after low-complexity reads were removed. With CosmosID's (CosmosID Inc., Rockville, MD) bioinformatics platform, concatenated, unassembled sequencing reads were directly analyzed (Hasan *et al.*, 2014). The prodigal software was used for open reading frame (ORF) prediction of the assembled contigs. Bowtie package was run for variant detection and digital gene expression in different sample groups (Langmead, 2010). Paired-end reads were assigned to samples based on their unique barcode by using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH). Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.9.1, http://qiime.org/scripts/split libraries fastq.html) quality control process. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). For each representative OTU, uparse software (V7.0.1001, http://drive5.com/uparse) was used to annotate taxonomic information (Edgar, 2013).

Annotation Analysis

The OTUs were performed for clustering and species taxonomic analysis. The core genus was defined as average relative abundance >0.1% (Huang *et al.*, 2018). The alpha and beta diversity were analyzed by the quantitative insight into microbial ecology (QIIME2) software package (Ran *et al.*, 2021). Usually, five metrics were calculated, and Chao1 indices were used for abundance analysis; the observed-species metric represents the number of distinct OTUs (Operational Taxonomic Units) found in each group; Shannon and Simpson indices revealed the community diversity and evenness; good-coverage revealed the sequencing depth index (Eckburg *et al.*, 2005). To access the variation in species complexity among samples, beta diversity analyzed by QIIME software (Version 1.9.1) was carried out. Cluster analysis was preceded by principal component analysis (PCA) conducted to reduce the dimensionality of the original variable. The ade4 package and ggplot2 package in R software (Version 2.15.3) were utilized for this purpose (Santillan *et al.*, 2022).

RESULTS

Sequencing data quality assessment

In this study, five groups of samples were subjected to metagenomic next-generation sequencing and a total of 1,209,462 raw sequences were collected. After comparison with the reference database, a total of 1,182,298 high-quality effective sequences with an average length of 419-426 bp were finally selected after filtering. All sequences were then clustered at the 97% sequence similarity value, resulting in a total of 1,482 OTUs for final analysis.

Composition analysis of the sample microbiome

A total of 44 phyla, 26 classes, 225 orders, 320 families, 494 genera, and 228 species were found using the OTU annotations. To understand the microbial composition of the *Artemia* nauplli, as determined by

the phylum level analysis, 96% of all microorganisms in the samples belonged to three dominant phyla (relative abundance >1%). Although Proteobacter (58.68%) was the most dominant microorganism in all groups, its relative abundance in the FB group was much lower than in the other four groups (>86%), while the Fimicutes (26.54%) were significantly higher than in the other four groups (*Figure 1*). As shown in *Figure 2*, at the genus level, the dominant bacterial genus was Vibrio, Donghicola, Marinomonas, Alteromonas, Flavobacterium, and Photobacterium in all the tested groups. The relative abundance of identifiable bacterial genus in the FB group was much higher than in the other four groups. The relative abundance of Vibrio (15.8%) and Donhicola (12.53%) accounted for only 28.33% of all microorganisms in the FB group, while these two genera made up more than 55% in the control groups and in the FA and FC groups. In addition, the identifiable Streptococcus (17.13%) and Haemophilus (8.68%) were much higher than in the other groups. The core genera shown by heatmap revealed differences among the FB group and the other two groups, including the control group (*Figure 3*). 22 of 35 genera belonged to Proteobacteria, while the Bacteroidota, Firmicutes, Actinobacteria, Cyanobacteria, and Bdellovbrionota processed 5, 4, 2, 1 and 1 genera respectively. Huge differences were found among the FB group and the other four groups, as shown in the heatmap; 14 genera were strikingly enriched in the FB group compared with the other groups ($P<0.05$). Compared with the bacterial abundance in the other groups, 15 genera were significantly depleted in the FB group. Moreover, the heatmap showed that the abundance of potential pathogens (Vibrio) in the FB group was significantly distinguished from other groups, indicating that treatment with TCCA would significantly alter the bacterial composition in *Artemia* nauplii.

Alpha diversity analysis of the microbiomes

The Good's Coverage revealed the sequencing depth, closer to 1, the true value of the sequencing data. The sequencing results of each group were reliable, and

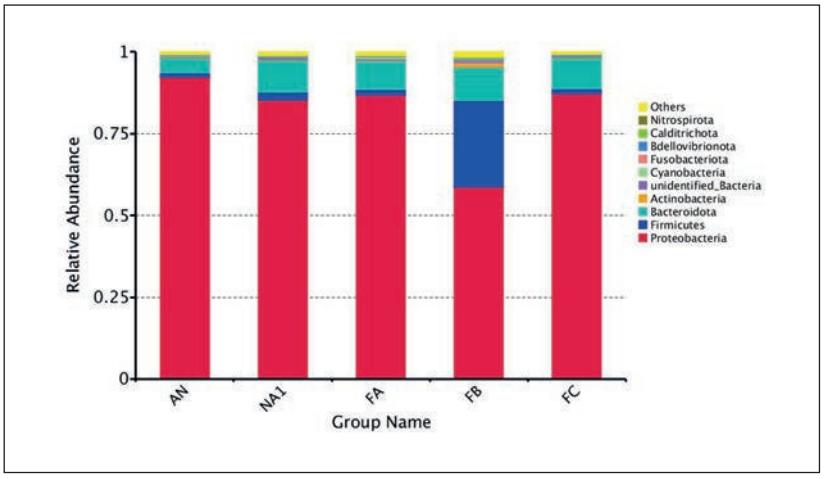

Figure 1 - *Species abundance histogram at the phylum level (Top 10).*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5 ppm TCCA treated group; FB: 1ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

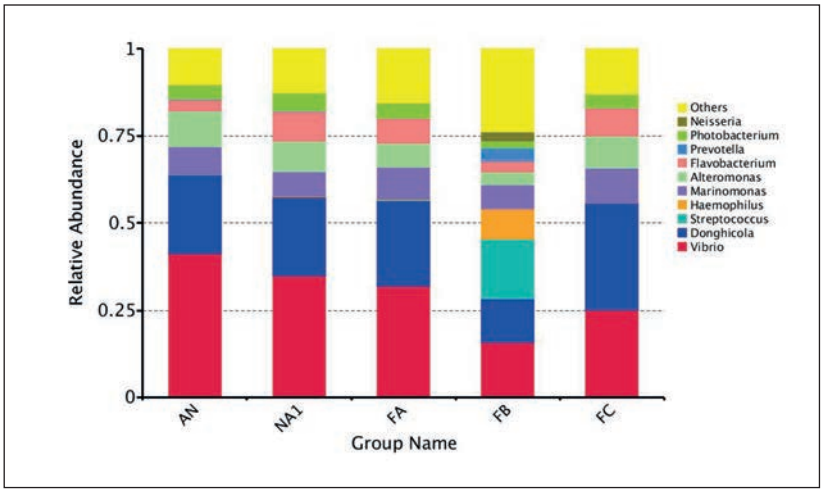

Figure 2 - S*pecies abundance histogram at genus level (Top 10).*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5 ppm TCCA treated group; FB: 1ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

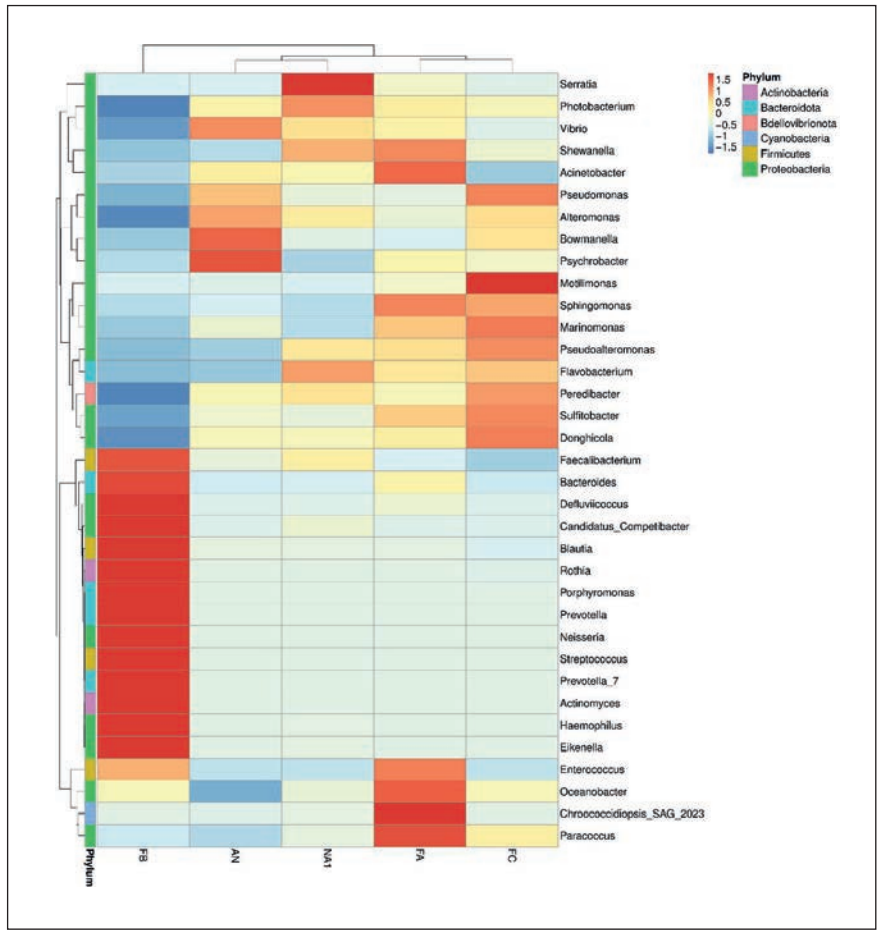

Figure 3 - *Relative abundance of core genera shown by heatmap.*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5 ppm TCCA treated group; FB: 1 ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

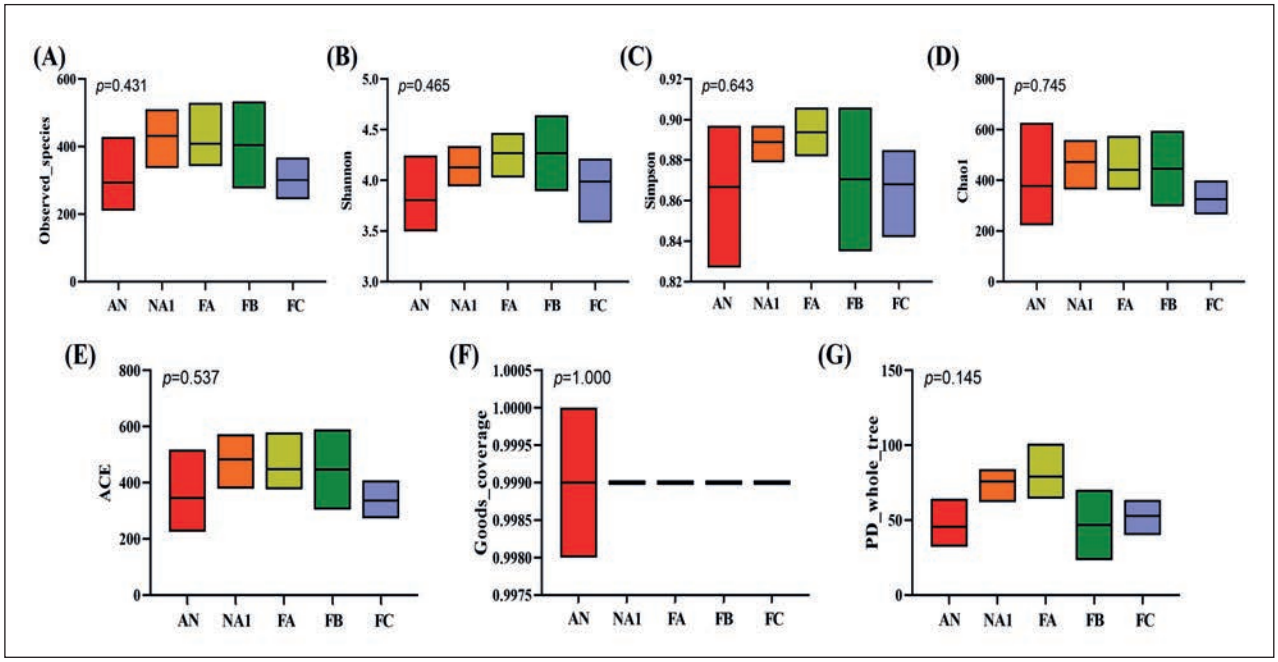

Figure 4 - *Alpha diversity index of each group.*
(A) The number of observed species; (B) Shannon index of species diversity; (C) Simpson index of species diversity; (D) Chao1 index of species richness; (E) ACE index of species richness; and (F) Good-coverage index; and (G) phylogenetic tree index.

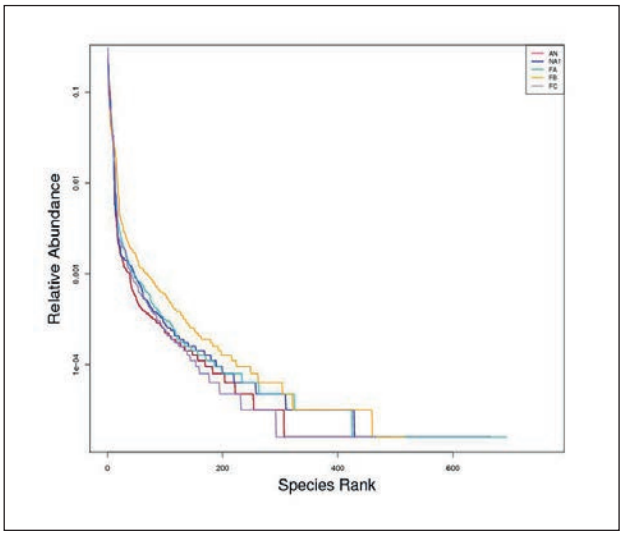

Figure 5 - *The rank-abundance analysis.*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5ppm TCCA treated group; FB: 1 ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

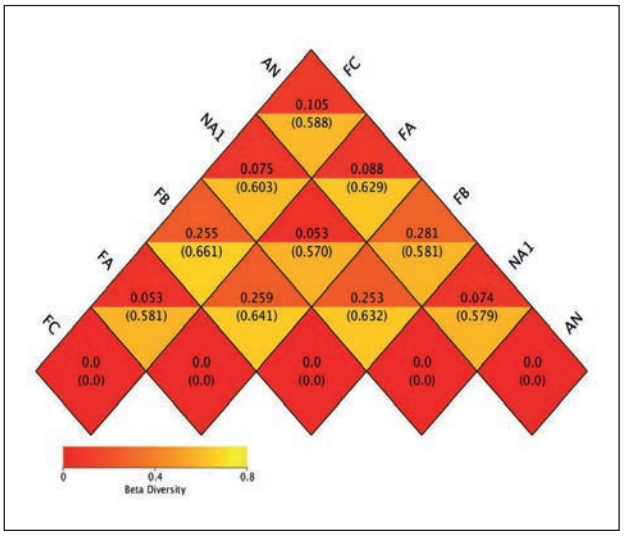

Figure 6 - *Beta diversity heatmap based on the UniFrac distance.*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5 ppm TCCA treated group; FB: 1ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

the sequencing depth was sufficient to represent the majority of microbe species (*Figure 4*). As shown in *Figure 4*, bacterial community richness was expressed in the Chao 1 and ACE indices, and the diversity of bacterial communities was expressed in the Shannon and Simpson indices, yet no significant difference was found in the different groups ($p>0.05$). This phenomenon indicates that different treatments have no significant effect on the richness and diversity of *Artemia nauplii*. The Rank-abundance distributions (RADs) were used to assess the relative abundance of established genera and to ascertain whether there were differences among each tested group. As presented in *Figure 5*, the rank-abundance curve of the FB group was gentler than in the other four groups, indicating that the species distribution of FB was more diverse and even; meanwhile, no significant difference was found in the FA, FC and control groups.

Beta diversity analysis of the microbiomes

To compare the diversities of the microbiomes in different groups, beta diversity analysis was performed. Beta diversity measurements are depicted in the heatmap (*Figure 6*), and the numbers in the grid represent the coefficient difference among all the tested groups. The smaller the value, the narrower the difference among the samples. According to the results, the weighted-UniFrac distance of groups ranged from 0.00 to 0.281, while unweighted-UniFrac distance varied from 0.00 to 0.661, suggesting that the species diversity of the FB group was clearly different from that of other groups. PCA analysis showed that samples were aggregated according to different treatments (*Figure 7*). The spatial distance difference between the FB group and other groups is significant, indicating that the microbial composition of the FB group was significantly different from that of other groups.

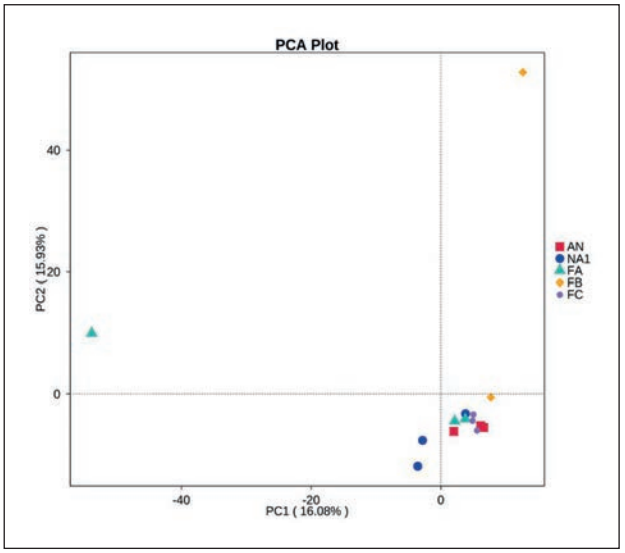

Figure 7 - *PCA of microbial community.*
AN: the unsterilized control group; NA1: the unsterilized control group washed with sterilization water; FA: 0.5 ppm TCCA treated group; FB: 1 ppm TCCA treated group; FC: 1.5 ppm TCCA treated group.

DISCUSSION

As an important microbial feed supplement, *Artemia* nauplii has been widely used for delivering diverse nutrition to larval stages of aquatic animals in the aquaculture industry (Gomez-Gil *et al.*, 1998). The quality of *Artemia* nauplii directly affects the survival and growth of the fish and shellfish larvae (Martello *et al.*, 2000). However, the microorganism compositions of *Artemia* nauplii were unclear and no research was focused on it. In this study, NGS was carried out to determine the exact microorganism compositions of nauplii.

As we know, the *Artemia* cysts were sterilized before running through the hatching process; however, the hatching of *Artemia* cysts under nonsterile conditions usually results in high production of bacteria (Arun *et al.*, 2017). The high level of pathogenic bacteria in nauplii might lead to the outbreak of disease in shrimp farms. In this study, an extra sterilization process was performed before the nauplii were fed to the shrimp larvae. Three different concentrations of TCCA were used for nauplii sterilization, after which metagenomic next-generation sequencing was carried out to determine the microorganism composition in different TCCA treated groups.

In this research, two groups were used as controls, namely the AN group and NA1group. Unlike in the AN group, the nauplii in the NA1 group were washed several times with sterilized water, and a slight difference was found between the two control groups. Species abundance at the genus level showed that after washing with sterilized water, the Vibrio in nauplii was reduced by 6.26%, indicating that the extra wash step would help to reduce Vibrio in nauplii. The addition of this wash step would be necessary before feeding to shrimp larvae.

TCCA has been extensively used as a disinfectant due to its chlorinating and oxidizing properties. As expected, the species abundance in TCCA treated groups was quite different from that in the control groups. Compared with the control groups, Vibrio abundance in TCCA treated groups descended to a certain degree. The Vibrio in TCCA treated groups were reduced by 9.35% (FA group), 25.55% (FB group) and 16.3% (FC group), respectively. Obviously, differences were found among the TCCA treated groups. According to the sequencing results, Vibrio abundance at the genus level in the FB group, namely the 1.0ppm TCCA treated group, was 15.8%, which was obviously lower than the 0.5 ppm group and 1.5ppm group (32.02% and 25.05%, respectively). Suggesting that the 1ppm of TCCA might be the best working concentration for sterilization in nauplii, the higher concentration of TCCA did not further reduce the Vibrio in nauplii. It is reasonable to speculate that low concentrations of TCCA were insufficient to kill and inhibit the propagation of pathogenic bacteria such as Vibrio. Clearly, the more the better was not suitable for the TCCA used as disinfectant in nauplii. The 1.5 ppm TCCA did not further reduce Vibrio proliferation: on the contrary, a high concentration of TCCA led to high abundance of Vibrio in *Artemia* nauplii compared with the 1.0 ppm TCCA treated group. Presumably, the high concentration of TCCA killed some of the bacteria that competed for survival with Vibrio, consequently resulting in the propagation of Vibrio. Therefore, more work should be done to figure out the best sterilization time for more reasonable pre-treatment of *Artemia* nauplii before feeding to shrimp larvae.

In conclusion, pre-treatment with TCCA at 1ppm concentration for 25 min would greatly reduce a fetal pathogen such as Vibrio in *Artemia* nauplii, offering an alternative to antibiotics and promote the healthy development of the shrimp farming industry.

Funding

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Ethic statement

The sample collection and experiments in the study were in compliance with the Laboratory Animals recommendations (Ministry of Science and Technology of China 2006) and approved by the Animal Ethics Committee of Zhejiang Institute of Freshwater Fisheries.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest in this work.

Data availability

The data used to support the findings of this study are included in the article.

Acknowledgments

None

Data availability statement

All data included in this study are available upon request by contacting the corresponding author.

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