Dietary *Chlorella vulgaris* Improves Growth and Modulates Gut Microbiota in Giant Freshwater Prawn (*Macrobrachium rosenbergii*)

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Abstract

The giant freshwater prawn (*Macrobrachium rosenbergii)* is a commercially vital species in global aquaculture due to its rapid growth and high market demand, and *Chlorella vulgaris* is a nutrient-rich microalga lauded for its potential as a sustainable feed supplement. In this preliminary study, the effects of *C. vulgaris* supplementation on the growth and gut microbiota of giant freshwater prawn was investigated. Prawns were randomly assigned to three groups and fed diets supplemented with *C. vulgaris* at 0 g/kg (control), 30 g/kg (T1), or 60 g/kg (T2) for 14 days. For growth performance analysis, body weight was measured in a randomly selected 10% of the population at the beginning, midpoint, and end of the experiment. For gut microbiota analysis, faecal samples were taken from *M. rosenbergii* and used for DNA extraction and amplification via PCR. The PCR product was sequenced using 16S rRNA sequencing on Illumina platform. Results showed significantly improved growth rates in the supplemented groups, with T2 (highest growth rate) having 55% higher weight gain compared to the controls. Gut microbiota analysis revealed increased diversity, with the alpha biodiversity indices showing higher indices in supplemented groups compared to the controls although non-significant (p = 0.196, 0.136, 0.532 and 0.304 for Ace Chao1, Shannon and Simpson respectively). Specifically, the T1 group (highest alpha biodiversity) exhibited the following increases compared to the control: Ace by 53.55%, Shannon by 61.16%, Chao1 by 55.94%, and Simpson by 55.90%. In β-biodiversity analysis, there were shifts in bacterial community composition, with a notable increase in beneficial bacteria such as *Synechocystis* spp. and decrease in potentially pathogenic ones such as *Chitinibacter* spp. in the supplemented groups. PICRUSt2 analysis predicted enhanced metabolic pathways related to vitamin synthesis and energy metabolism, suggesting improved gut health and nutrient utilization. Although some results lacked statistical significance due to the short study duration, the findings suggest that long-term *C. vulgaris* supplementation may yield more pronounced benefits in *M. rosenbergii* aquaculture, potentially improving growth performance.

Keywords: *Chlorella vulgaris*, *Macrobrachium rosenbergii*, gut microbiota, growth performance, aquaculture

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INTRODUCTION

The giant freshwater prawn (*Macrobrachium rosenbergii*) has become a highly valued species in global aquaculture, with production reaching 313,756 tonnes and a market value exceeding $2.45 billion in 2021 with China leading the industry, accounting for 54.4% of global production, followed by Bangladesh, Thailand, Myanmar, and India (Pillai and Panda, 2024). Despite being native to Malaysia and the country's historical role as an early innovator in prawn aquaculture, Malaysia's annual output remains comparatively low at 189.1 metric tonnes, equivalent to RM15.5 million in 2023 (DOF, 2023). This contrasts sharply with the species' premium market price (RM68–90/kg) and its recognized potential to drive rural economic development (Pillai *et al.*, 2022). The disparity between Malaysia's natural endowment

and its modest production underscores substantial untapped potential for expanding sustainable prawn farming and enhancing local livelihoods.

Industrial intensification in *M. rosenbergii* aquaculture has amplified disease risks, with bacterial pathogens (*Vibrio* spp., *Aeromonas hydrophila*), viral agents (white tail disease virus), and fungal infections (*Fusarium solani)* thriving in high-density systems (Ahmed *et al.,* 2023). Poor water quality exacerbates pathogen spread, causing mortality rates of 60–90% in uncontrolled outbreaks. To mitigate losses, antibiotics like oxytetracycline and florfenicol are routinely added to water or incorporated into feed as prophylactics to prevent the introduction and establishment of bacterial infections; as metaphylactics to control the spread of infection within a population when some individuals are already affected; or as therapeutics to treat animals that are clinically diseased (De Souza *et al*., 2020). While effective in the short term, this practice has led to alarming resistance patterns: 90% of aquatic bacteria now resist at least one antibiotic, and 20% exhibit multidrug resistance (An *et al*., 2023). Critically, bacteria's ability to transfer antibiotic resistance genes to human pathogens, therefore posing a global One Health threat (Pepi and Focardi, 2021).

Chlorella vulgaris is a microalgal species which can be found in diverse aquatic and terrestrial environments, from slow-moving water bodies up to high alpine environments (Aigner *et al.,* 2020). It is one of the most widely used microalgae in aquaculture, applied both as a direct feed source and as a functional additive in diets for various species (Sukri *et al.,* 2016). For commercial purposes, it is grown in outdoor ponds or photobioreactors where conditions like light, nutrients, pH, and temperature are optimized to maximize biomass and the production of valuable compounds (Panahi *et al.,* 2019). It is rich in essential macronutrients such as protein, vitamins, pigments, and the so-called *Chlorella* Growth Factor (CGF) (Ajiboye *et al.,* 2012). Notably, *Chlorella* contains beta-1,3-glucan, an immunostimulant known to reduce blood lipids, scavenge free radicals, and enhance the immune response of *M. rosenbergii* against *Aeromonas hydrophila* infection (Sahoo *et al.,* 2008). Dietary immunostimulants like *Chlorella* offer both economic and sustainability benefits by reducing reliance on costly antimicrobials and mitigating the risk of antimicrobial resistance (Kela, 2022). Supplementation with *C. vulgaris* has also been shown to improve growth performance, digestibility, digestive enzyme activity, and antioxidant capacity in multiple aquaculture species (Eissa *et al.,* 2023). Therefore, this study evaluates the effects of two levels of *Chlorella* supplementation on the growth and gut microbiota of *M. rosenbergii*.

MATERIALS AND METHODS

Ethical Approval

Animal use was approved by the Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (UPM) under certificate No.UPM/IACUC/AUP-R045/2024.

Study Period and Location

This study was conducted at Aquatic Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia from 10 October 2024 to 23 October 2024.

Experimental Animal and Feeding Trial

Ninety giant freshwater prawns were acquired from Klang, Selangor, with the initial weight of 10±2 g**,** and were 3 months old. The prawns were randomly placed in 42 x 28 x 30 cm glass aquaria, with 10 prawns in each tank. The prawns were acclimatized in clear glass aquaria for 2 weeks before the feeding trial began. During the acclimation period, the prawns were fed with commercial shrimp and prawn diet (Star Feedmills (M) Sdn. Bhd., Selangor, Malaysia) without any added supplements until visual satiety was observed. Each tank was provided with aeration to maintain the dissolved oxygen level at around 7–9 ppm, measured using YSI multiparameter probe (YSI Incorporated, Ohio, USA) and the temperature was maintained at around 24 °C in an air-conditioned room with a 12-hour light and 12-hour dark photoperiod

throughout the experiment. Overhead filter boxes were used for mechanical and biological filtration.

For the feeding trial, the prawns were divided into three groups, comprising of 0 g/kg (control), 30 g/kg (T1) and 60 g/kg (T2), each containing 30 prawns. The prawns were fed commercial pellets infused with *C. vulgaris* according to the dosages for 14 consecutive days. The *C. vulgaris* powder was obtained from Algae Living Sdn. Bhd, (Perak, Malaysia). The dry *C. vulgaris* powder was combined with sterile distilled water at the concentration of 0.3 g/ml. For T1 (30 g/kg *C. vulgaris* supplementation), 100 mL of the *C. vulgaris*-infused distilled water was sprayed onto 1 kg of pellet, and for T2 (60 g/kg *C. vulgaris* supplementation), 100 mL of the *C. vulgaris*-infused distilled water was sprayed onto 1 kg of pellet (Maliwat *et al*., 2020; Eissa *et al*., 2023). The pellets were then left to dry in a cool, dry place. The nutrient content of the commercial pellet and *C. vulgaris* powder, determined using nutrient proximate analysis following AOAC guideline 2016 (AOAC, 2016), was included in Table 1. The prawns were fed until satiation twice a day, once in the morning and once in the afternoon.

Water quality parameters, especially dissolved oxygen, ammonia, and nitrate were regularly monitored throughout the experiment using YSI multiparameter probe (YSI Incorporated, Ohio, USA). Nitrite concentrations were measured using the API Freshwater Master Test Kit (API Fishcare, Pennsylvania, USA). Ammonia levels were maintained at 0 parts per million (ppm), nitrite at approximately 0–0.5 ppm, and nitrate at approximately 0–10 ppm throughout the experimental period. For *M. rosenbergii*, the safe range of ammonia, nitrite and nitrate are below 8.45 mg/L, 1mg/L and 32mg/L respectively (Motta *et al.*, 2024; Hickey, 2013; Mallasen and Valenti, 2006). Biweekly water changes of 30% were performed, with replacement water prepared by storing and aerating tap water for 24 hours to remove chlorine. Feed residue, faecal materials and moulted shells were removed daily to maintain water quality. PVC pipes were provided as shelters to reduce stress and prevent cannibalism, particularly during moulting phase.

Growth Performance

The growth performance was measured morphometrically, by randomly taking the body weight of 10% of the population at the beginning, midpoint, and at the end of the feeding trial. The growth performance metrics were derived using the following calculations:

Weight gain = (final weight – initial weight)/initial weight ×100%..... (1)

Specific growth rate = (ln final weight – ln initial weight)/days ×100%..... (2)

Sample Collection

At the end of the 14-day feeding trial, six prawns were randomly selected from each group for sampling, as mentioned by Zhang and Sun, 2022. Euthanasia was performed using MS-222 (Syndel, USA), following a modified protocol based on Darbyshire *et al*. (2019). The prawns were immersed in dechlorinated tap water containing MS-222 at a concentration of up to 20 mg/L, monitored closely until all thoracopod movement ceased, indicating complete anaesthesia (Darbyshire *et al.*, 2019). Most of the prawns took up to 20 minutes to be euthanised.

For gut microbial sampling, the euthanised prawns were sterilised externally using 70% ethanol. Then, the prawns were dissected dorsally using sterilised scissors and forceps to expose the intestine. The intestine was isolated and removed from the body. The faecal material was carefully extracted from the intestine using aseptic technique**.** The faecal material underwent DNA extraction using the commercial ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, California, USA) following manufacturer's protocol.

Library Preparation and Sequencing

The 16S rRNA V3 hypervariable regions were amplified from gDNA using the primer pair forward 341F (CCTACGGGNGGCWGCAG) and reverse 785R (GACTACHVGGGTATCTAATCC) (Klindworth *et al*., 2013; García-López *et al.*,

2020). An additional five bases of inline barcode and partial Illumina adapter were incorporated at the 5' end of the primers to enable inline barcoding (Glenn *et al.*, 2019). Different samples were amplified using different combinations of the forward and reverse inline primers. PCR was carried out using REDiant II PCR Master Mix (Apical Scientific, Malaysia) with the following thermal cycling profile: initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 30 seconds. The PCR product was visualised on 1.5% agarose gel, and purified using Solid Phase Reversible Immobilisation (SPRI) beads at the volume of 0.8 times the gDNA sample volume. The purified amplicons were used as the template for eight cycles of index PCR to incorporate the complete Illumina adapter and Illumina-compatible dual-index barcodes. The constructed libraries were subsequently size-selected using SPRI beads at the volume of 0.8 times the gDNA sample volume pooled into a single tube. Quantification of the pooled libraries was performed using DeNovix dsDNA HighSensitivity Assay (Denovix, Delaware, USA). Sequencing of the pooled libraries was performed on NovaSEQ6000 platform using the 2×150 bp paired-end sequencing configuration (Illumina Inc., San Diego, CA, USA).

Data Analysis

Demultiplexing and primer trimming of the raw paired-end reads used cutadapt version 1 (Martin 2011). The trimmed reads were subsequently merged using fastp version 0.21 (Chen *et al.* 2018). The processed reads were imported into Quantitative Insights into Microbial Ecology (QIIME 2) version 2023.9 (Bolyen *et al.* 2019) and denoised into amplicon sequence variants (ASVs) using Denoising Algorithm for Amplicon Data **(**DADA2) version 1.26.0 (Callahan *et al.* 2016). Taxonomic assignment of the ASV using QIIME2-feature-classifier that has been trained on the latest Greengenes2 database (McDonald *et al.* 2023). ASVs (amplicon sequence variants) with taxonomic assignment to at least the phylum level was selected for subsequent analysis. Following data rarefaction to the sample with the lowest number of reads, alpha diversity analysis was conducted in QIIME2. Alpha diversity indices, including Chao's richness estimator (Chao1), abundance-based coverage estimator (ACE), Shannon diversity index (Shannon), Simpson Diversity Index (Simpson) were calculated to assess within-sample diversity. For beta diversity analysis, distances based on the Jaccard similarity index were computed using the rarefied dataset.

In addition, both the ASV table and taxonomic classification table were exported using QIIME2 tools into tab-separated values (TSV) format and manually formatted to generate input compatible with Microbiome Analyst (Chong *et al*. 2020). The output can be used to perform SparCC co-occurrence network construction (Friedman and Alm, 2012) and statistical analysis using Linear discriminant analysis (LDA) Effect Size (LEfSe) method (Segata *et al.* 2011). The filtered relative abundance table was also used as the input to generate Krona plots for intuitive exploration of relative abundances within the hierarchies of taxonomic classifications (Ondov, Bergman, and Phillippy 2011). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) version 2.5.2 was used to predict the functional profiles of each ASV, and the resulting data were subsequently visualised and statistically analysed using Statistical Analysis of Metagenomic Profiles (STAMP) software (Douglas *et al.* 2020; Parks *et al.* 2014). The growth performance and alpha diversity of gut microbiota were calculated using Kruskal–Wallis H test in SPSS version 27.0 at statistical significance level of $p = 0.05$. For the predicted metabolic pathways, one-way ANOVA was performed, followed by Tukey–Kramer test using STAMP software.

RESULTS AND DISCUSSION

Growth Performance

The analysis of the morphometric data of body weight gain showed that there was a significant difference between the prawns

supplemented with *C. vulgaris* (T1 and T2) and those not supplemented. However, there was no significant difference in specific growth rate between those supplemented and not supplemented. There was also no significant difference in weight gain percentage between both supplemented groups. However, there was no significant difference in weight gain and specific growth rate between the T1 and T2 groups (Table 2).

Table 1. Nutrient proximate analysis for *Chlorella vulgaris* powder and commercial pellet

Parameters	***Chlorella vulgaris***	**Commercial pellet**
Dry Matter (%)	90.4	90.4
Moisture (%)	9.6	9.6
Ash (%)	11.3	9.3
Crude Protein (%)	52.6	32.7
Crude Fat (%)	1.5	1.2
Crude Fibre (%)	0.1	3.6
Gross Energy (J/g)	16,440	17,134

Table 2. Growth performance parameters of *Macrobrachium rosenbergii* supplemented with *Chlorella vulgaris* meals administered during a feeding trial of 14 days

Parameters	**Control (Mean ± SD)**	**T1 (30 g/kg) (Mean ± SD)**	**T2 (60 g/kg) (Mean ± SD)**	***p*-value**
Initial weight (g)	10.17 ± 0.65	10.22 ± 1.92	9.33 ± 1.66	1.35
Final weight (g)	11.61 ± 3.13^{a}	15.67 ± 3.00^{b}	14.89 ± 3.82^{b}	0.03
Weight gain (%)	14.59 ± 32.21^{a}	57.28 ± 39.39^{b}	70.49 ± 77.17^{b}	0.07
Specific growth rate (% day^{-1})	0.341 ± 2.228^{a}	3.318 ± 2.483^{b}	5.843 ± 3.203^{b}	0.148

Superscript letters indicate significant differences between groups.
Kruskal-Wallis was used to determine significant differences in Alpha diversity index values between groups.

Table 3. Alpha diversity measures for the bacterial population in *Macrobrachium rosenbergii* gastrointestinal tracts

Parameters	**Control (Mean ± SD)**	**T1 (Mean ± SD)**	**T2 (Mean ± SD)**	***p*-value**
Ace	63.684 ± 20.321	97.786 ± 10.064	96.836 ± 9.892	0.196
Chao1	63.784 ± 20.087	99.466 ± 12.727	97.976 ± 11.095	0.136
Shannon	1.586 ± 0.911	2.556 ± 0.314	2.222 ± 0.976	0.532
Simpson	0.458 ± 0.256	0.714 ± 0.089	0.616 ± 0.290	0.304

Kruskal-Wallis was used to determine significant differences in Alpha diversity index values between groups.

Table 4. Percentage of relative abundance of the most dominant bacterial phylum in all groups based on analysis performed on Microbiome Analyst platform

Treatment	***Firmicutes*_D**	***Proteobacteriota***	***Actinobacteriota***	***Cyanobacteria***
Control	58.53%	40.76%	0.57%	0.03%
Treatment 1	33.01%	48.62%	11.04%	6.68%
Treatment 2	43.19%	44.08%	2.32%	9.40%

Table 5. Percentage of relative abundance of the most dominant bacterial genus in all groups based on analysis performed on Microbiome Analyst platform

Treatment	*Enterobacteriaceae* members	*Lactococcus* spp.	*Mycoplasmatales* members	*Alphaproteobacteria*
Control	27.33%	36.04%	22.20%	8.14%
Treatment 1	21.36%	20.51%	3.31%	22.63%
Treatment 2	13.68%	26.39%	11.18%	27.81%

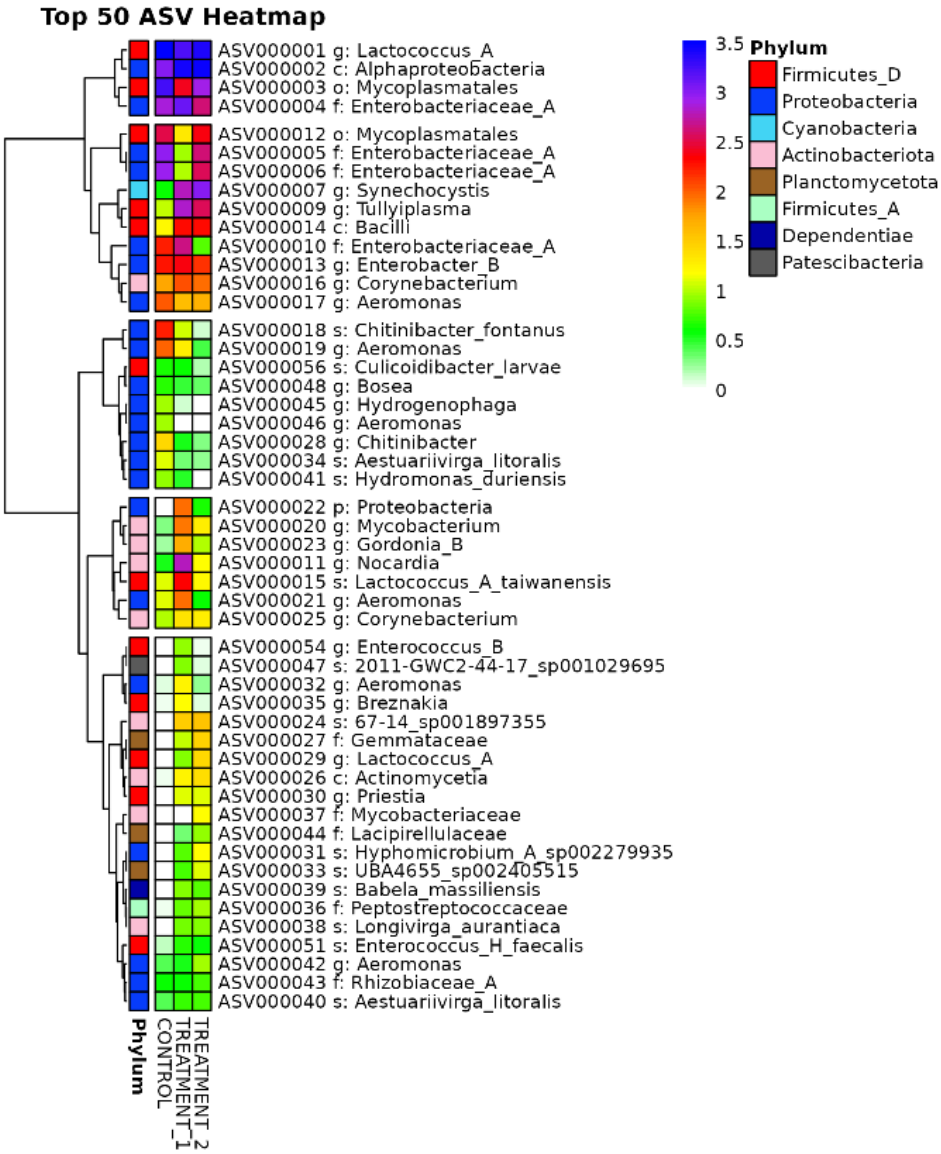

Figure 1. Heatmap plot of the most abundant ASV identified across groups. The y-axis shows ASVs that were clustered based on Ward2 hierarchical clustering method. ASVs were annotated and coloured based on their taxonomic affiliation at the phylum level. The heatmap scale indicates the average relative abundance of ASV by groups (normalized to 10,000 reads/sample) in a 10-based logarithmic scale.

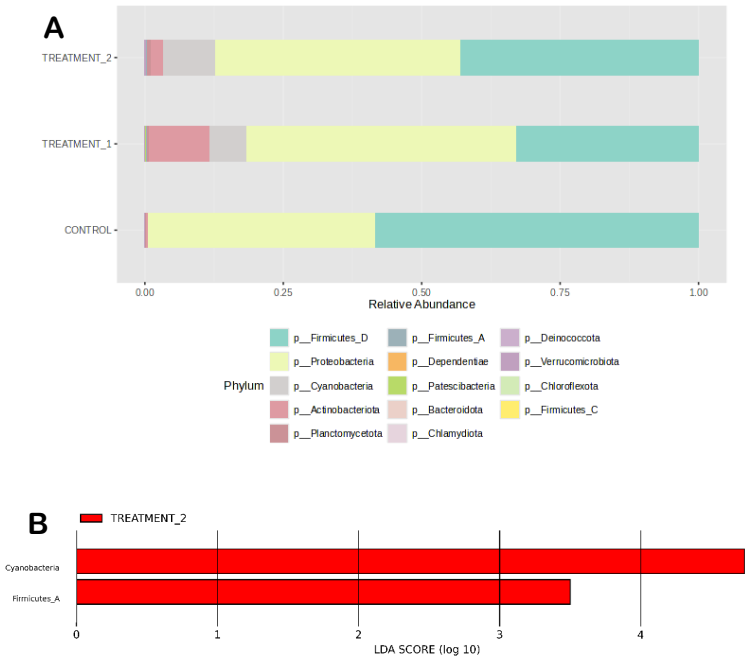

Figure 2. (A) Stacked bar plot showing the average relative abundance of bacterial phylum per group. (B) Identification of Differentially Abundant phylum Across Groups Using LEfSe. The taxa were considered significantly different if its relative abundance showed a statistical significance with *p*-value less than 0.01 and a logarithmic LDA score of more than 3.0.

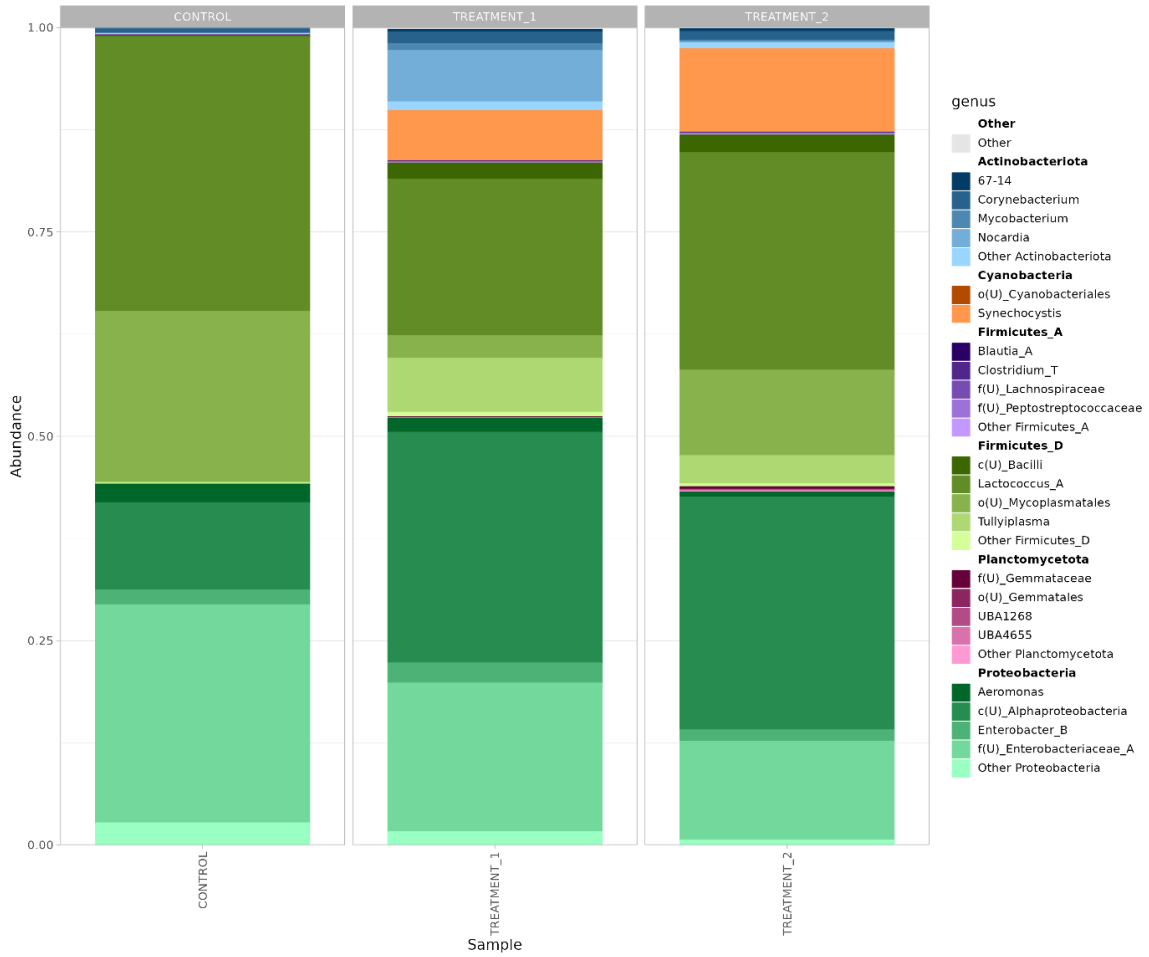

Figure 3. Stacked bar plot showing the average relative abundance of bacterial genus per group.

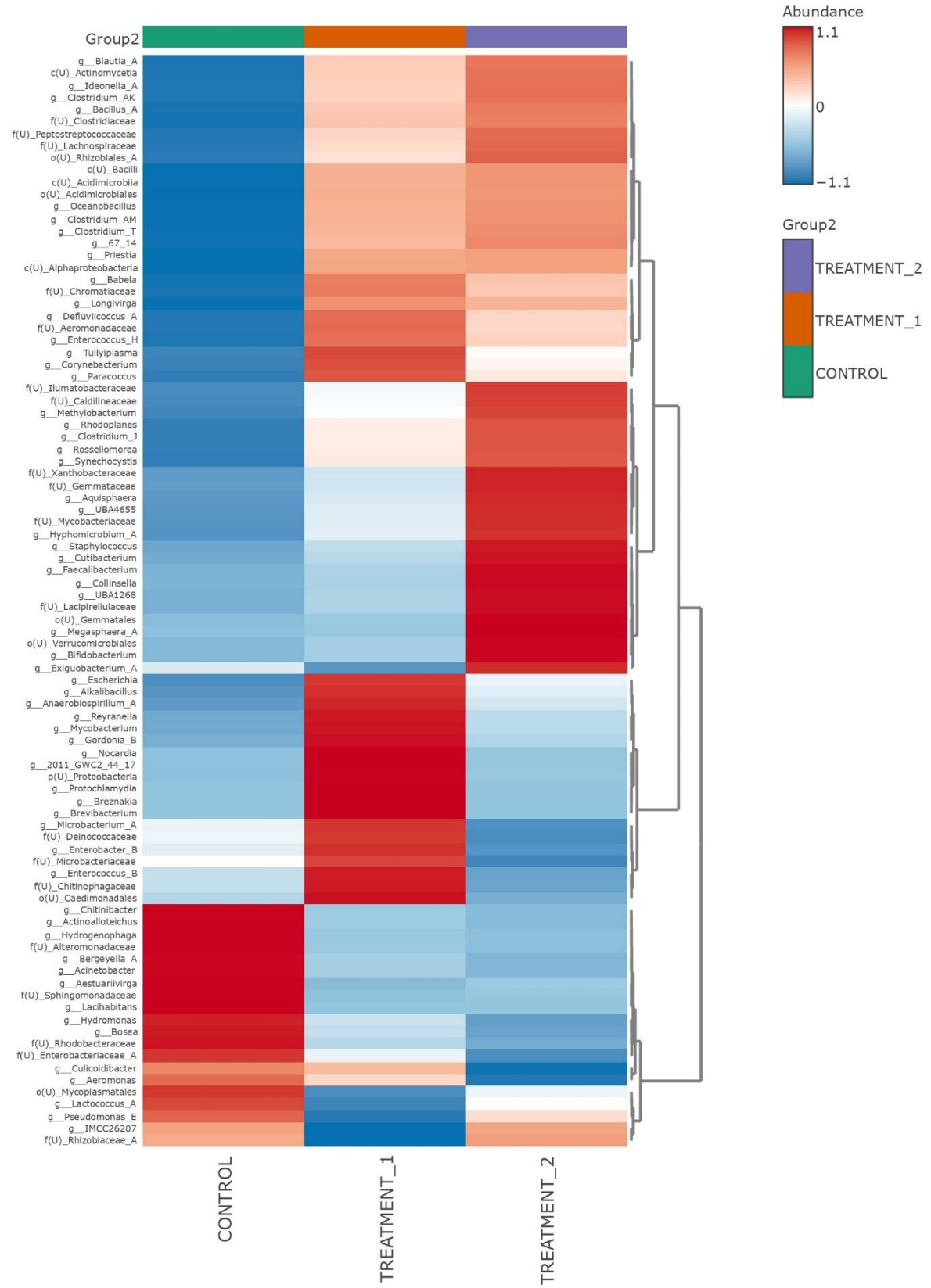

Figure 4. Heatmap of bacterial abundance in control, T1 and T2 groups based on Euclidean distance. Colour intensity of the heatmap represents the abundance/absence of the bacterial genus in the sample group.

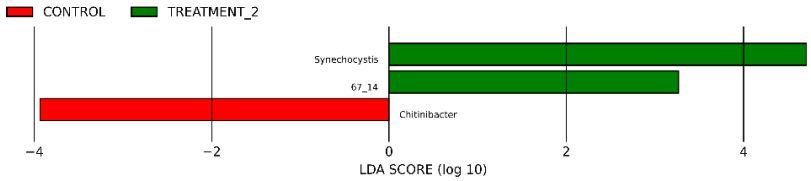

Figure 5. LEfSe analysis reveals significant differences in bacterial genera between control and treatment groups in *Macrobrachium rosenbergii*. The results are displayed using LDA scores (log 10).

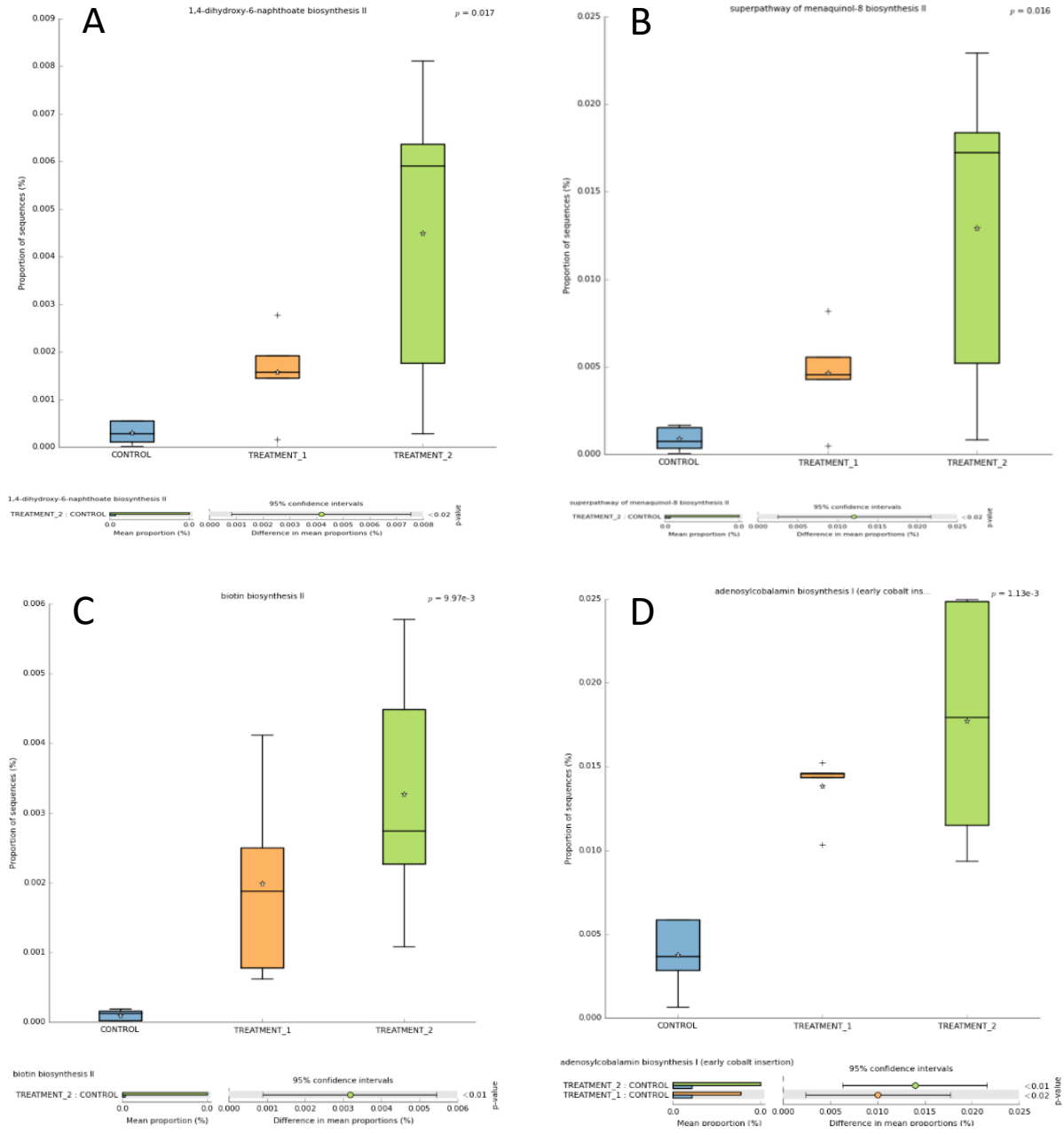

Figure 6. Boxplot showing the proportion of sequences of (%) of four active predicted metabolic pathways using STAMP software. One-way ANOVA was performed to check for significant difference, followed by Tukey-Kramer for the post-hoc test. (A) 1,4-dihydroxy-6-naphthoate biosynthesis II (B) superpathway of menaquinol-8 biosynthesis II (C) biotin biosynthesis II (D) adenosylcobalamin biosynthesis I (early cobalt insertion).

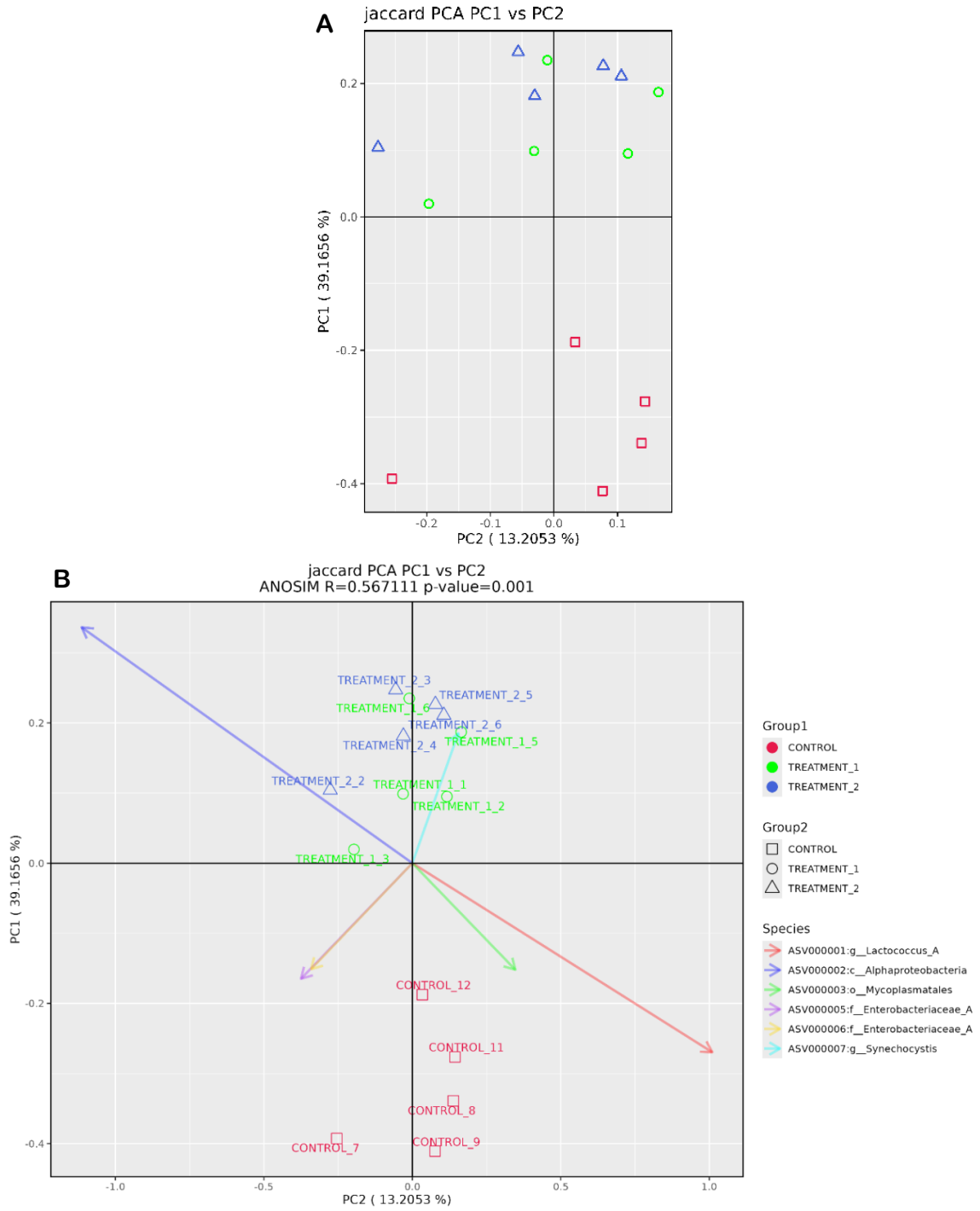

Figure 7. (A) Beta diversity as assessed through Principal Coordinate Analysis (PCoA) plots representing two dimensions: PC1 vs. PC2. Distances between samples were determined using Jaccard similarity index, capturing compositional differences in microbial communities. Red square represents Control group, green circles represent T1 group, and blue triangles represent T2 group. Samples clustering closely together on the plots were indicative of shared microbial compositions, while those situated farther apart reflect greater dissimilarity in community structure. (B) Detailed Principal coordinate analysis (PCoA) biplot (PC1 vs PC2) based on Jaccard similarity index. Amplicon Sequence Variants (ASVs) significantly associated with the clustering and positions of samples on the PCoA were overlaid as vectors with length of each vector representing the magnitude of influence exerted by the corresponding ASV on the axis.

Alpha and Beta Biodiversity

The Chao1, ACE, Shannon, and Simpson indices—all of which represent the individual gut microbiota diversity, indicated that T1 had the highest diversity of gut microbiota, followed by T2 and control group. However, none of the alpha diversity tests showed significant difference among the three groups ($p > 0.05$) (Table 3).

For beta diversity analysis, which describes about the difference in gut microbiota composition between samples, principal coordinate analysis (PCoA) was performed to compare the community structure among treatments, based on Jaccard similarity index, to evaluate the bacterial profiles associated with the intestines of prawns after supplementation with *C. vulgaris*. The Jaccard-based PCoA revealed significant restructuring of gut microbial communities across experimental groups (Analysis of Similarity (ANOSIM) R = 0.567, $p < 0.05$). The ordination demonstrated distinct spatial clustering patterns where treatment samples (both T1 and T2) formed cohesive clusters in the ordination space. In contrast, control samples exhibited clear spatial segregation, notably greater intra-group dispersion compared to the treatment samples. The samples from the treatment groups were observed to be heavily influenced by the class *Alphaproteobacteria***,** while the control group samples were most heavily influenced by the genus *Lactococcus***.**

A heatmap of the top 50 most abundant ASVs was plotted (Figure 1), consisting of eight Phyla: *Firmicutes* A, *Firmicutes* D, *Proteobacteria*, *Cyanobacteria*, *Actinobacteriota*, *Planctomycetota*, *Dependentiae*, and *Patescibacteria*. It can be observed that *Lactococcus*, *Alphaproteobacteria*, *Mycoplasmatales*, and *Enterobacteriaceae* were dominant in all groups. *Synechocystis*, *Tullyipasma*, and *Bacilli* were more abundant in the treatment groups compared to the control group, whereas *Chitinibacter* and *Aeromonas* were more abundant in the controls.

At the Phylum level**,** *Firmicutes* D, *Proteobacteria*, and *Actinobacteriota* constantly predominated across all groups. However, the treatment groups were distinguished by the presence of *Cyanobacteria* and the reduction ofrelative abundance of *Actinobacteriota* (Table 4 and Figure 2). LEfSe analysis showed that only *Cyanobacteria* and *Firmicutes* A were significantly more enriched in the T2 group, whereas for the other Phyla, there were no significant differences among the groups.

At the Genus level (Table 5 and Figure 3), control group gut microbiota was characterised by a predominance of members of the *Enterobacteriaceae* family (27%), *Lactococcus* spp. (36%), and members of the *Mycoplasmatales* order (22%). In contrast, T1 group was characterised by a predominance of members of the *Alphaproteobacteria* class (23%), members of the *Enterobacteriaceae* family (21%), and *Lactococcus* spp. (21%). The T2 group exhibited a similar pattern with members of the *Alphaproteobacteria* class (28%), members of the *Enterobacteriaceae* family (14%), and *Lactococcus* spp. (26%) as the most abundant taxa. *Tullyiplasma* (6%) and *Nocardia* spp. (7%) were the most abundant in the T1 group, while *Synechocystis* (9%) was highest in the T2 group. Members of the *Mycoplasmatales* order were less abundant in the T1 and T2 groups (3% and 11%, respectively) compared to the control group (Table 5).

A heatmap of clustered heatmap was also created at the Genus level, based on Euclidean distance (Figure 4). It can be observed a greater diversity and relative abundance of bacterial Genus were present in the treatment groups compared to the control group. LEfSe analysis was conducted to identify differential taxa between the control and supplemented groups at the Genus level (Figure 5).

LEfSe analysis detected two primary differentially abundant taxa between the control and Treatment 2 (6% *C. vulgaris* supplementation) groups (Figure 5). The *Chitinibacter* genus showed a significant negative LDA score (-3.98) in the control group, indicating its higher abundance in control specimens. Conversely, *Synechocystis* and an unidentified taxon from the *Solirubrobacterales* family, 67_14 exhibited positive LDA scores (approximately

4.71 and 3.26, respectively) in Treatment 2, suggesting their enrichment in the *C. vulgaris*-supplemented group.

Metabolic Pathways Predictions

Analysis of the predicted metabolic pathways was performed on PICRUSt2 at Kyoto Encyclopaedia of Gene and Genomes (KEGG) level 3 based on the metadata obtained from the 16S rRNA sequencing of gut microbiota. A total of 390 pathways were identified, with 28 showing significantly different expression levels between the supplemented groups and the control group. Of these 28 pathways, 27 were significantly upregulated in the supplemented groups. A few of the prominent pathways are shown in Figure 6. The results demonstrate a significantly higher abundance of these pathways in Treatment 2 (6% *C. vulgaris* supplementation) compared to the control group **(***p* = 0.017, *p* = 0.016, *p* = 9.97^{-3}**,** and *p* = 0.029**,** respectively). Treatment 1 (3% *C. vulgaris*) showed intermediate values between the control and Treatment 2, suggesting a dose-dependent response to *C. vulgaris* supplementation.

Growth Rate

In the present study, there was a significantly improved growth rate in supplemented prawns compared to those that were not supplemented. *C. vulgaris* is known to be an effective supplement that promotes growth (Maliwat *et al.,* 2020). Sukri *et al*. (2016) also mentioned that *C. vulgaris* supplementation of up to 10% promotes the growth of *M. rosenbergii* without any detrimental effects. *C. vulgaris* has also been shown to support growth in various other aquaculture species, such as olive flounder (*Paralichthys olivaceus)* and Pacific white shrimp (*Litopenaeus vannamei)* (Rahimnejad and Lee, 2016; Maliwat *et al.,* 2016). The bioactive compounds found in *Chlorella*, including *Chlorella* growth factors and significant levels of macronutrients like proteins and lipids, enhance the growth rate of *M. rosenbergii* in this experiment (Badwy *et al.,* 2013). The notable growth improvement observed in prawns was also attributed to the high digestibility and concentration of active growth promoters within *Chlorella* cells (Khani *et al.,* 2017). Furthermore, *M. rosenbergii* post-larvae utilise *Chlorella* in their diets more effectively than other aquatic organisms (Amaya *et al.,* 2007). In the present study, it was also observed that the T2 group has a slightly higher growth rate compared to T1. This may be related to the level of *C. vulgaris* inclusion, although longer study is needed to confirm this.

Gut Microbiota

Alpha diversity analysis (Chao1**,** ACE**,** Shannon and Simpson indices) revealed an increase in diversity in the supplemented groups. However, the differences were not statistically significant (Table 3). This outcome was likely influenced by the short duration of the feeding trial, which lasted only 14 days. It is possible that extending the feeding period would have resulted in a more pronounced increase in alpha diversity within the supplemented groups. A study on Pacific white shrimp (*Litopenaeus vannamei*) demonstrated that *Chlorella* supplementation enhances gut microbial diversity and richness, in a study that investigated the effect of different stocking densities on the ability of *L. vannamei* to utilise *C. sorokiniana* during an 8-week feeding trial (Yuan *et al.,* 2024). Another study conducted also found that in juvenile Nile tilapia (*Oreochromis niloticus*) fed with 0.5% and 2% *C. vulgaris* and sampled at day 15 and day 30, the alpha diversity only increased at 30 days of feeding (Huang *et al.,* 2023).

Beta diversity analysis using Principal Coordinates Analysis (PCoA) based on the Jaccard similarity index revealed distinct clustering patterns. Samples from both treatment groups were closely grouped together, whereas samples from the control group were separated from the supplemented groups and exhibited greater dispersion among themselves (Figure 7). ANOSIM R value of 0.567 indicates moderate to strong separation between groups, and high intra-group similarity, demonstrating that *C. vulgaris* supplementation changes the gut microbiota population, making it distinct from the control group.

Most of the dominant Phyla found in all experimental groups—*Proteobacteria*, *Actinobacteriota*, *Firmicutes*, and *Cyanobacteria*—are bacteria commonly found in the intestines of cultured *M. rosenbergii* (Xu *et al*., 2023), except *Cyanobacteria*. *Cyanobacteria*, especially *Synechocystis* spp., which was found to be significantly abundant in the T2 group (6% inclusion), is a recognised probiotics that have been used as supplements in Pacific white shrimp (*L. vannamei*) and black tiger shrimp (*Penaeus monodon*), and was proven to be helpful in immunity against white spot syndrome virus (WSSV) (Zhai *et al*., 2019). *Firmicutes* A, which was significantly more abundant in the T2 group, was known to play a significant role in carbohydrate metabolism in the gut of prawns, and the presence of *Firmicutes* was also associated with high growth performance in *M. rosenbergii* (Lan *et al.*, 2023). Hence, the increased abundance of *Firmicutes* in the T2 group coincides with elevated growth rates (Table 2), suggesting a potential functional linkage between this taxon and enhanced growth performance in *M. rosenbergii*. In a study involving the use of *C. sorokiniana* as the main protein source for *L. vannamei* in an 8-week feeding trial, mentioned there was a correlation between the levels of *Chlorella* inclusion with the presence of *Firmicutes* (Yuan *et al*., 2023). However, no literature has been found on the correlation between the level of *Chlorella* inclusion with the presence of *Synechocystis* spp.

On Genus level, it can be observed that the relative abundance of *Enterobacteriaceae* family decreases with the inclusion of *C. vulgaris*. A few members of the *Enterobacteriaceae* family detected were *Klebsiella spallanzanii*, *Enterobacter* spp., and *Salmonella* spp. *Enterobacteriaceae* are normal flora of the gut, which can be found in healthy animals (Prakash and Karmagan, 2013; Fikri *et al*., 2022). However, in the case of dysbiosis, which may occur when animals are stressed, these bacteria can become pathogenic and cause disease. Some *Enterobacter* species, such as *E. cloacae*, were known to cause growth retardation in *M. rosenbergii* (Gao *et al*., 2024).

Lactococcus spp. also exhibited reduced relative abundance in treatment groups T1 and T2 compared to the control group. *Lactococcus* spp. is one of the major lactic acid bacteria (LAB) in the shrimp gut (Zhao *et al*., 2018). They are known for their probiotic potential, and *L. lactis* has been proven to improve growth rate, feed utilisation, and immune function in *M. rosenbergii* (Kader *et al*., 2021). However, some species such as *L. garvieae* are known to be serious disease-causing bacteria in aquaculture species, leading to fatal septicemia, including in *M. rosenbergii* (Angeles *et al*., 2009).

Other than that, *Mycoplasmatales* was also seen to be reduced in the treatment groups compared to the control group. *Spiroplasma* spp., a member of the *Mycoplasmatales* order, has been studied for its role in causing diseases in prawns and shrimps (Liang *et al*., 2011).

Another dominant bacterial group, *Alphaproteobacteria* Genus, were more abundant in the supplemented groups compared to the non-supplemented group. Hoang *et al*. (2020) mentioned that *Alphaproteobacteria* presence in the shrimp gut could reduce the prevalence of pathogens, leading to a high survival rate in the culture system. Thus, it is believed that an increased *Alphaproteobacteria* population resulted in better gut health and eventually better nutrient optimisation.

Based on the LEfSe analysis (Figure 5), *Chitinibacter* was significantly higher in the control group compared to the other groups. *Chitinibacter* has been researched for its negative impact on the peritrophic matrix (PM) and gut immunity (Liu *et al*., 2024). According to the same author, this is due to its ability to degrade chitin, an important component of the PM. Its presence can lead to a decrease in *Lactobacillus* populations, which are known to be beneficial to the host.

Predicted Metabolic Pathways

The supplemented groups exhibited increased abundance of key metabolic pathways, ncluding superpathway of menaquinol-8 biosynthesis, 1,4-dihydroxy-6-naphthoate biosynthesis, biotin biosynthesis II, and

adenosylcobalamin biosynthesis I (early cobalt insertion) pathways.

Menaquinone biosynthesis pathways indicate increased potential for menaquinone (vitamin K_2) biosynthesis in the gut microbiota of prawns receiving *C. vulgaris* supplementation. Menaquinone plays a crucial role in electron transfer systems in prokaryotes, which is essential for microbial energy metabolism (Zhi *et al*., 2014).

Juvenile Chinese white shrimp (*Fenneropenaeus chinensis*) supplemented with vitamin K reported significant increase in weight gain (Shiau and Liu, 1994). The upregulation of the 1,4-dihydroxy-6-naphthoate biosynthesis pathway also suggested an increased presence of bacteria capable of synthesizing menaquinone precursors (Zhi *et al*., 2014), which correlates with elevated menaquinone production.

Biotin, also known as vitamin B7, is essential for various metabolic processes including carbohydrate and fatty acid metabolism, protein synthesis, cell growth and development and energy production at the cellular level, since it is an important coenzyme for a few carbon dioxide-fixing enzymes, *e.g.* pyruvate carboxylase and acetyl-CoA carboxylase (Shiau and Chin, 1998). Shiau and Chin (1998) also observed improved growth rate in *P. monodon* supplemented with biotin.

Vitamin B12, including adenosylcobalamin as its cofactors, is a vital micronutrient that plays an important role in multiple physiological processes, (Medagoda and Kyeong, 2025). The same author also reported that optimal inclusion of vitamin B12 in *P. vannamei* diet increases growth rate and improves various physiological processes such as innate immunity, tissue vitamin B12 deposition, haematopoiesis, antioxidant activity, ammonia stress tolerance, fatty acid synthesis, and methionine metabolism. In salmonids, insufficiency of vitamin B12 resulted in disrupted growth and development, reduced appetite, inefficient feed utilization, megaloblastic anaemia, fragmented red blood cells, irregular blood profiles, lethargy, stunted growth, darkened coloration, and nervous system dysfunction (Liu *et al*., 2022).

CONCLUSION

This preliminary study on different levels of *C. vulgaris* supplementation in *Macrobrachium rosenbergii* shows promising results, with trends of improved growth and increased gut microbiota diversity. Supplementation increased beneficial bacteria and decreased potentially pathogenic ones, suggesting enhanced metabolic pathways for vitamin synthesis and energy metabolism. Longer-term studies may reveal more pronounced benefits, potentially improving growth and disease resistance.

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AUTHORS' CONTRIBUTIONS

AN was responsible for the study design, conceptualization, experimental work, data analysis, and manuscript preparation. NDMT, AS, MNAA, NI, and NMN provided supervision throughout the study. All authors have read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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