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Shaping the Aquatic Microbiome: Bacillus Probiotics Drive Community Succession and Enhance Nitrogen Cycling in *Litopenaeus vannamei* Culture Water

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ABSTRACT

This study elucidates the ecological and functional impacts of Bacillus-based probiotics on microbial community dynamics and water quality in *Litopenaeus vannamei* culture systems. A controlled seven-day experimental framework was employed to compare probiotic-amended and untreated tanks through integrated water quality assessment and high-throughput 16S rRNA gene sequencing. In probiotic-treated water, ammonium concentrations exhibited a consistent decline concomitant with nitrate accumulation, reflecting the enhancement of nitrification and overall nitrogen biogeochemical cycling. Microbial community analyses revealed marked compositional restructuring, characterized by the temporal proliferation of copiotrophic and fast-growing heterotrophic taxa, including *Vibrio*, *Pseudoalteromonas*, and *Alteromonas*. While Bacillus supplementation effectively reduced the relative abundance of potentially pathogenic *Vibrio* and favored the enrichment of functionally beneficial genera such as *Halomonas* and *Acinetobacter*, it also promoted opportunistic successional trajectories within the microbial assemblage. Overall, the results indicate that single-strain Bacillus probiotics can improve water quality through stimulation of nitrogen transformation processes, but may simultaneously trigger unpredictable community-level restructuring, emphasizing the need to consider whole-community responses and to develop multi-strain probiotic formulations for achieving more stable and resilient shrimp aquaculture ecosystems.

1. INTRODUCTION

Aquaculture, particularly shrimp farming, is one of the most dynamic sectors in global food production and is increasingly regarded as central to rural development and food security [1]. It provides essential protein at a time when capture fisheries are constrained by overexploitation and climate-driven pressures that threaten long-term sustainability. Within this sector, the whiteleg shrimp (*Litopenaeus vannamei*) has become globally dominant due to its rapid growth, high fecundity, and tolerance to a wide range of environmental and salinity conditions [2]. These attributes make *L. vannamei* the cornerstone of modern shrimp aquaculture, supporting export economies in Asia and Latin America while generating employment across the production value chain. Moreover, its cultivation contributes to global sustainability targets, including Sustainable Development Goals (SDG) 2 (Zero Hunger) and 14 (Life Below Water).

Despite its economic importance, the intensification of shrimp farming has generated substantial ecological and managerial challenges. High stocking densities, limited water exchange, and excessive feeding practices lead to the accumulation of organic matter and nitrogenous wastes, which deteriorate water quality, destabilize microbial communities, and promote pathogen proliferation [3]. Nutrient enrichment often results in ammonia accumulation and the formation of toxic metabolites, causing physiological stress and reduced survival of cultured shrimp. In addition, the discharge of nutrient-rich effluents into surrounding waters contributes to eutrophication, algal blooms, and oxygen depletion, ultimately disrupting aquatic ecosystems and threatening coastal biodiversity and fisheries [4].

To mitigate these problems, chemical treatments and antibiotics have traditionally been employed in shrimp

aquaculture. While effective in the short term, their extensive use has raised serious concerns regarding environmental contamination, bioaccumulation, and the development of antimicrobial resistance [5]. Resistant pathogens can spread through aquatic environments and food chains, exacerbating disease outbreaks and undermining long-term sustainability. Although alternative mitigation technologies such as sediment removal, biofilters, biofloc systems, and ozone treatment are available, they are often costly, technically demanding, and inaccessible to small-scale farmers. Consequently, the reliance on antibiotics, coupled with limited affordable alternatives, highlights the urgent need for sustainable water-quality management strategies.

Probiotics have emerged as a promising biological approach to address these challenges. Probiotics are defined as live microorganisms that confer health or ecological benefits when applied in adequate amounts [6]. In aquaculture systems, probiotics have been reported to improve water quality, suppress pathogenic bacteria, enhance nutrient utilization, and stabilize microbial communities [7]. Among probiotic candidates, *Bacillus* species are particularly valued due to their spore-forming ability, tolerance to environmental stress, and diverse functional roles in organic matter degradation, nutrient cycling, and pathogen inhibition [8]. *Bacillus* spp. are capable of mediating nitrogen and phosphorus transformations, thereby mitigating eutrophication and improving overall ecosystem stability, while also antagonizing pathogenic *Vibrio* species [9, 10].

Nevertheless, growing evidence suggests that single-strain Bacillus applications may not fully address the complexity of aquaculture ecosystems. Probiotic supplementation can induce microbial succession and, under certain conditions, favor fast-growing opportunistic taxa [11]. Since microbial communities in aquaculture systems are structured through complex ecological networks, the introduction of Bacillus-based probiotics inevitably influences microbial diversity, succession, and ecosystem functioning. Understanding these ecological responses is therefore essential for designing effective and sustainable probiotic-based management strategies.

From the microbial ecological perspective, the introduction of probiotics represents not only a functional intervention but also a biological disturbance capable of reshaping community structure and interaction networks. According to post disturbance succession theory, alterations in resource availability and competitive hierarchies following microbial perturbation may favor r-strategist populations characterized by rapid growth and opportunistic behavior, potentially at the expense of K-strategist taxa that contribute to long-term stability and functional redundancy. In aquaculture environments, such r/K selection processes can lead to transient dominance of copiotrophic and potentially pathogenic groups, including members of *Vibrio* and other Gammaproteobacteria, thereby generating unintended ecological consequences alongside water-quality improvement. Increasing evidence indicates that single-strain probiotic applications may reduce community evenness, promote opportunistic succession, and modify trophic coupling within microbial networks, ultimately influencing ecosystem resilience. Despite these recognized risks, integrative studies that simultaneously resolve high-resolution microbial community succession and multivariate water-quality dynamics following probiotic addition, particularly under highly controlled laboratory water systems using high-throughput sequencing, remain limited. This knowledge gap constrains our ability to fully evaluate the dual ecological effects of probiotics, namely their capacity to enhance biogeochemical functions while potentially destabilizing microbial community structure.

This study aims to investigate the ecological roles of Bacillus-based probiotics in intensive *L. vannamei* culture systems, with a specific focus on microbial community dynamics and water quality. Using controlled experimental treatments, high-throughput 16S rRNA gene sequencing, and comprehensive water-quality monitoring, this research evaluates how probiotic supplementation influences aquatic microbial structure and functional processes. By emphasizing the culture water environment rather than host responses, this study provides clearer insights into probiotic–microbiome interactions and contributes to the development of sustainable, microbiome-based approaches for shrimp aquaculture management.

2. MATERIALS AND METHODS

2.1 Experimental design

This study was conducted as a short-term (7-day) controlled experiment under laboratory conditions using only water as the culture medium for *Litopenaeus vannamei*. No substrate, biofloc, or sediment matrix was included; therefore, the observed effects of the Bacillus-based probiotic represent responses within a simplified aquatic system rather than those occurring in complex pond environments (Figure 1). Such simplified experimental designs are commonly applied to isolate microbially driven processes and to minimize confounding environmental variables in aquaculture ecology studies [12, 13].

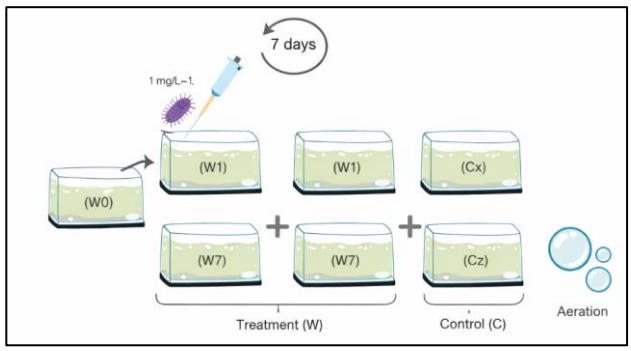

Figure 1. Experimental design

High-throughput bacterial community profiling was performed on the probiotic-treated group, with the initial time point prior to probiotic addition (W0) serving as the pre-disturbance baseline representing the original microbial assemblage before ecological perturbation. Thus, W0 provides an internal control for evaluating temporal community succession induced by probiotic amendment. Sequencing of the parallel control tanks was not conducted due to logistical and financial limitations in sequencing capacity, and the experimental design therefore prioritized resolving high-resolution successional trajectories within the perturbed system rather than full between-treatment community comparisons. The absence of control-group sequencing restricts direct statistical contrasts of community composition;

however, interpretation of probiotic effects was strengthened by integrating (i) the W0–W1–W7 successional sequence as a within-system ecological gradient and (ii) comparative analyses of key water quality parameters between control and probiotic treatments over time. This combined framework allows functional responses in nitrogen dynamics and system stability to be evaluated in relation to observed microbial shifts, while acknowledging that causal attribution of specific taxa-level changes should be interpreted within the context of baseline-referenced succession and physicochemical divergence rather than strict control treatment community contrasts [14].

The experiment was specifically designed to evaluate short-term ecological responses to probiotic supplementation; consequently, no substrate addition, feeding, or other management interventions were applied. During the experimental period, shrimp were not provided with additional feed, and technical constraints, including limited sequencing depth and the lack of control-group community profiling, are recognized as inherent limitations that may influence the generalization of community-level causal inferences.

2.2 Bacillus-based probiotic and cultural conditions

The commercial probiotic QuickPro Direct used in this study contained a consortium of *Bacillus* species as stated on the product label, including *Bacillus subtilis, Bacillus licheniformis, Bacillus polymyxa, Bacillus megaterium, Bacillus coagulans, Bacillus cereus, Bacillus alvei, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans,* and *Bacillus firmus*. Such Bacillus-based multi-species probiotics are commonly applied in aquaculture to improve water quality and organic matter degradation [15, 16].

The product was activated by dissolving the recommended dose in sterile seawater and incubating under gentle aeration at 28–30°C for 6 h to enhance bacterial metabolic activity prior to application [17]. The activated suspension was enumerated using serial ten-fold dilutions followed by spread plating on appropriate agar media to determine viable cell concentrations (CFU mL^{-1}) [15]. The suspension was adjusted to obtain an operational concentration of 1×10^7 CFU mL^{-1}, a level reported to be effective for probiotic performance in shrimp culture systems [16].

Experimental application consisted of a single addition of the activated probiotic suspension at time zero to achieve a final dose of 1 mg L^{-1} (1 ppm) of the commercial product in the treatment tanks. Single-dose probiotic application has been widely used to assess short-term ecological responses and microbial succession in aquaculture water environments [17, 18].

2.3 DNA extraction and high-throughput sequencing

Water samples were collected aseptically from each tank and filtered through 0.2-µm pore-size polycarbonate membrane filters to concentrate microbial biomass [19]. Total genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol, incorporating mechanical disruption by bead beating and chemical lysis to ensure efficient recovery of bacterial DNA [20]. DNA concentration was quantified using a Qubit dsDNA HS fluorometer (Thermo Fisher Scientific), and DNA integrity was verified by agarose gel electrophoresis [21].

The V3–V4 hypervariable region of the 16S rRNA gene (~464 bp) was amplified using universal primers 341F and 805R [22]. PCR amplification consisted of an initial denaturation at 95°C for 3 min, followed by 25–30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. Amplicons were purified using AMPure XP magnetic beads and subjected to dual indexing using Nextera XT adapters following established protocols [23].

Sequencing libraries were quantified by qPCR, normalized, pooled, and sequenced on an Illumina MiSeq platform using 2 × 250 bp paired-end reads [24]. Raw reads were processed using Cutadapt to remove adapter and primer sequences [25]. Quality filtering, denoising, and chimera removal were performed using the DADA2 pipeline with the following parameters: truncLen = 240 for forward reads, and 200 for reverse reads, maxEE = 2 for both reads, truncQ = 2, and minLen = 200. After merging paired-end reads and removing chimeras, an average sequencing depth of approximately 35,000–50,000 high-quality sequences per sample was obtained.

To minimize biases caused by unequal sequencing depth among samples, the ASV table was rarefied to 30,000 sequences per sample prior to downstream diversity and community composition analyses. Amplicon sequence variants (ASVs) were inferred at single-nucleotide resolution using DADA2 [26], and taxonomic assignment was performed using a naïve Bayes classifier against the SILVA v138.1 reference database [27].

2.4 Water quality analysis

Water quality parameters measured in this study included temperature (°C), pH, dissolved oxygen (DO; mg L^{-1}), ammonium (NH_4^+–N; mg L^{-1}), nitrite (NO_2^-–N; mg L^{-1}), and nitrate (NO_3^-–N; mg L^{-1}), following standard practices in aquaculture water quality assessment [28]. Temperature, pH, and DO were measured daily using a multiparameter water quality checker (TOA WQC-22A).

Water samples were collected daily, homogenized, and stored in 250 mL polyethylene bottles prior to analysis. Samples were filtered and analyzed for NH_4^+–N, NO_2^-–N, and NO_3^-–N following Indonesian National Standard (SNI) methods [29]. Concentrations of nitrogenous compounds were determined using a Hitachi U-1900 UV–Vis spectrophotometer, as commonly employed in aquatic biogeochemical studies [30].

2.5 Statistical analysis

Water quality data are presented as mean ± standard deviation (SD) based on three replicate tanks per treatment (n = 3). Prior to multivariate analysis, data were tested for normality using the Shapiro–Wilk test and standardized by z-score transformation to minimize scale effects among variables [31]. Principal Component Analysis (PCA) was applied to reduce data dimensionality and to identify major gradients explaining variation in water quality parameters across treatments and sampling periods [32]. Relationships among water quality variables were examined using Pearson's correlation analysis for normally distributed data or Spearman's rank correlation for non-normal data [33]. All statistical analyses were performed using R software (version 4.5.0) with the packages FactoMineR and corrplot [34]. Statistical significance was accepted at $p < 0.05$.

For microbial community analysis, alpha diversity indices, including Shannon, Simpson, and Chao1, were calculated to

assess within-sample diversity using the phyloseq package in R. Beta diversity was evaluated based on the Bray–Curtis dissimilarity matrix and visualized by Principal Coordinates Analysis (PCoA) to compare microbial community composition among treatments and sampling times. All microbial community analyses were conducted using R software (version 4.5.0).

3. RESULT

3.1 Microbial community in Bacillus-based probiotic

The activated Bacillus-based probiotic (PB) at 1 ppm was dominated by the genus *Bacillus*, accounting for approximately 63% of the total relative abundance (Figure 2). Other dominant genera included *Paracoccus* (approximately 29%) and *Halomonas* (approximately 8%). This compositional profile confirms that the probiotic inoculum was functionally Bacillus-driven, characterized by Gram-positive, endospore-forming taxa known for ecological persistence and resistance to environmental stress [35, 36].

3.2 Analysis of microbial community

Across the sampling period from W0 (baseline) to W7 (post-treatment), the relative abundance of major bacterial taxa showed clear temporal changes as illustrated in the stacked bar plots (Figure 3(a)-(c)).

At the phylum level (Figure 3(a)), the microbial community was dominated by Pseudomonadota at all sampling times, and its relative abundance increased from W0 to W7. In contrast, the relative proportions of Bacteroidota, Actinobacteriota, and Firmicutes decreased over time, although they remained consistently present in the community.

At the family level (Figure 3(b)), Vibrionaceae, Rhodobacteraceae, Flavobacteriaceae, and Alteromonadaceae exhibited higher relative abundances at W1 and W7 compared with W0. Among these, Vibrionaceae showed a marked increase at W7. Rhodobacteraceae and Alteromonadaceae also increased in relative abundance at W1 and remained prominent at W7.

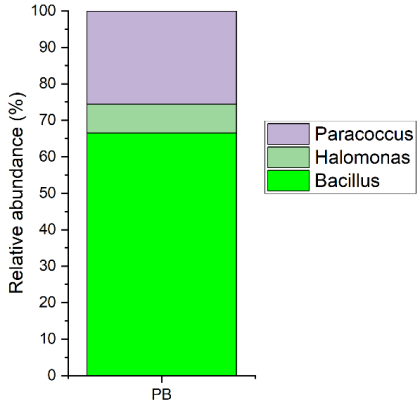

Figure 2. Relative abundance of bacterial genera in the activated Bacillus-based probiotic

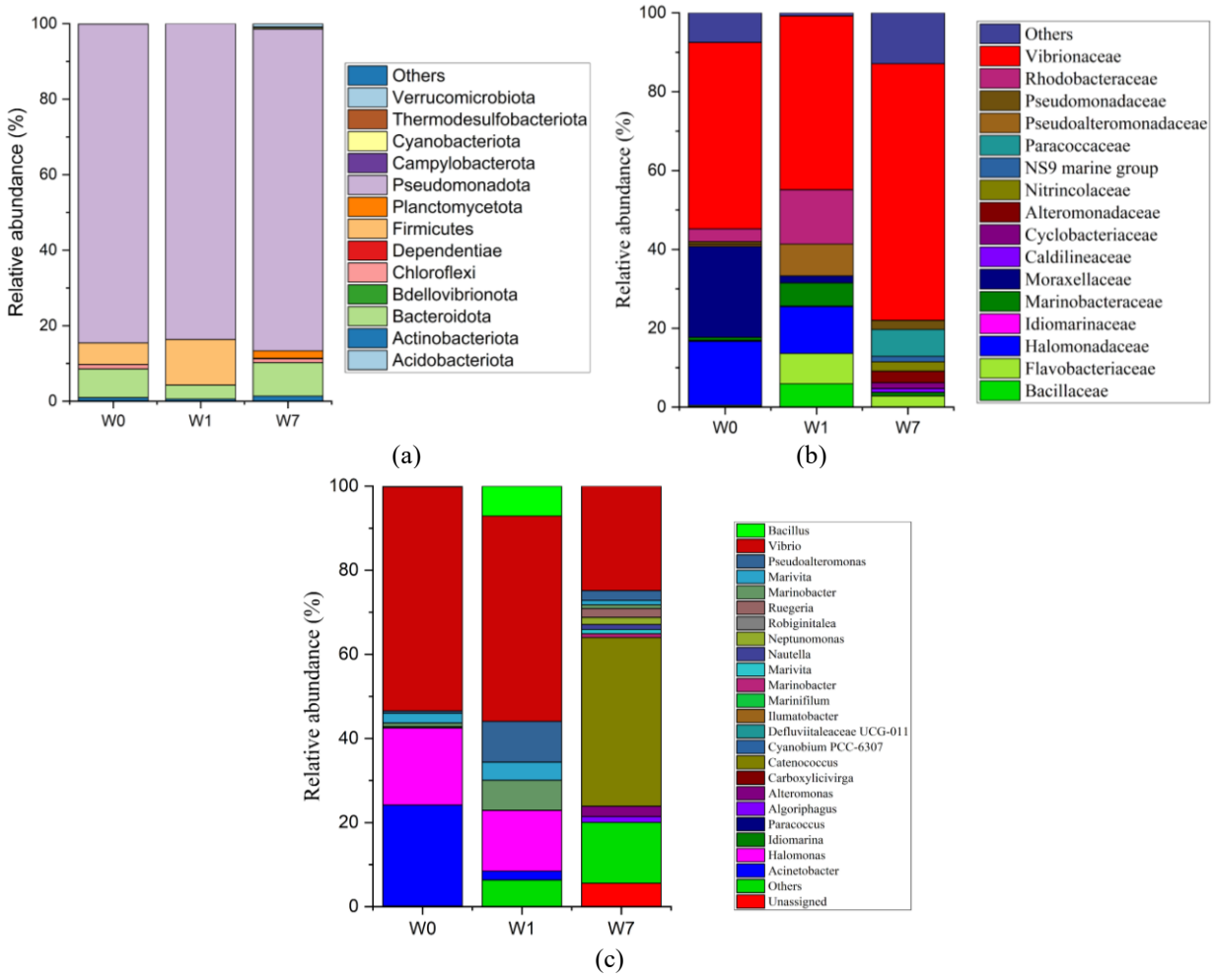

Figure 3. Relative abundance analysis of the water microbial community (a) Phylum level, (b) Family level, (c) Genus level

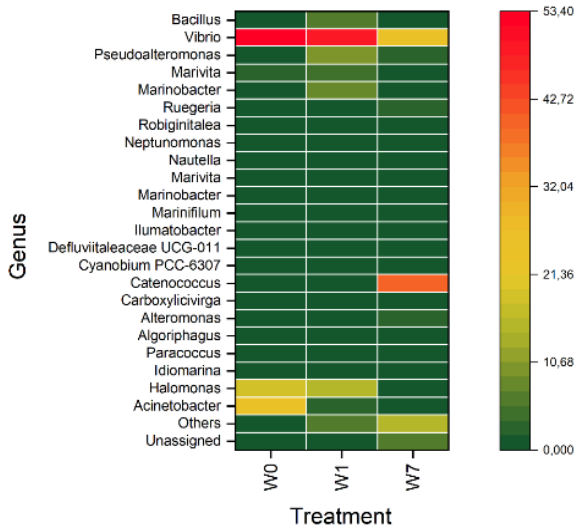

Figure 4. Genus-level heatmap of microbial community composition

At the genus level (Figure 3(c)), the microbial community at W0 was composed of a mixture of genera, including *Bacillus*, *Marivita*, *Ruegeria*, and *Pseudoalteromonas*. As the incubation progressed to W1 and W7, changes in community composition were observed. The relative abundance of *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, and *Marinobacter* increased at W1 compared with W0, whereas several genera that were relatively abundant at W0 decreased in proportion over time.

Figure 4 further illustrates the temporal shifts in dominant genera from W0 to W7. *Vibrio* and *Pseudoalteromonas* showed high relative abundance at the earlier sampling times and exhibited marked changes in abundance at W1 and W7. In addition, genera such as *Catenococcus*, *Halomonas*, and *Acinetobacter* were detectable at W7 and showed slightly higher relative abundance compared with earlier sampling times, although they did not dominate the community. Overall, these results indicate dynamic temporal changes in genus-level community structure rather than a uniform increase in abundance at later stages.

3.3 Water quality condition

Analysis of the experimental data encompassing treatments C0–C7 and W0–W7 demonstrated the effect of 1 ppm Bacillus probiotic inoculation on water quality parameters (Figures 5 and 6). Differences between control and probiotic treatments at the final sampling point (C7 vs W7) were statistically evaluated using an independent samples t-test for normally distributed variables or the Mann–Whitney U test for non-normal data, with significance accepted at $p < 0.05$.

Water temperature remained stable throughout the experiment, ranging from 28.0 to 28.5°C in both control and probiotic treatments (Figure 5(a)), with no significant difference between C7 and W7 ($p > 0.05$).

Dissolved oxygen (DO) levels increased during the early experimental period in both treatments and remained relatively stable thereafter (Figure 5(b)). Although the probiotic treatment tended to show slightly higher DO values than the control, no significant difference was detected between C7 and W7 on day 7 ($p > 0.05$). Similarly, pH values were consistently within the range of 7.6–7.8 across all treatments (Figure 5(c)), and no significant difference was observed between the control and probiotic groups at the end of the experiment ($p > 0.05$).

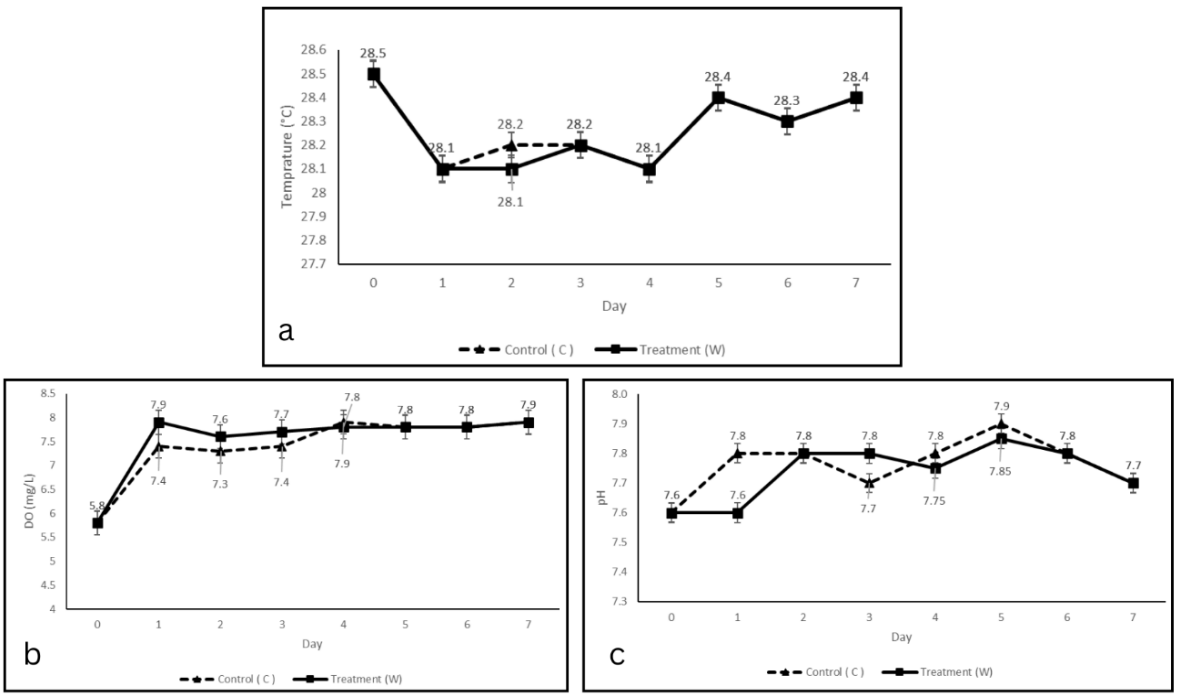

Figure 5. Water condition: The parameters of (a) Temperature, (b) Dissolved oxygen, and (c) pH

Marked differences were also observed in nitrogenous compounds (Figure 6). Ammonium (NH_4^+–N) concentrations in the control group remained relatively high, ranging from 2.381 to 3.984 mg L^{-1}, whereas in the probiotic treatment, NH_4^+–N decreased sharply from 3.984 mg L^{-1} at W0 to 1.290 mg L^{-1} at W7 (Figure 6(a)). Statistical comparison between C7 and W7 confirmed that this reduction was significant ($p < 0.01$).

Nitrite (NO_2^-–N) concentrations exhibited temporal fluctuations in both treatments (Figure 6(b)). The control group showed higher and less stable values, reaching a maximum of 0.415 mg L^{-1} at C4, whereas the probiotic treatment displayed a transient peak at W5 (0.596 mg L^{-1}) followed by stabilization at 0.514 mg L^{-1} by W7. A comparison between C7 and W7 revealed a statistically significant difference ($p < 0.05$).

Conversely, nitrate (NO_3^-–N) concentrations increased in the probiotic treatment, reaching 2.796 mg L^{-1} at W7, while remaining below 1.972 mg L^{-1} in the control throughout the experimental period (Figure 6(c)). The difference between C7 and W7 was statistically significant ($p < 0.01$).

Overall, statistical testing confirmed that Bacillus probiotic application significantly affected key nitrogen parameters (NH_4^+, NO_2^-, and NO_3^-) at the final sampling point, while temperature and pH remained unchanged. These results demonstrate that the visual trends observed in Figures 5 and 6 are supported by quantitative statistical evidence, particularly for enhanced ammonium removal and nitrate accumulation in the probiotic-treated system.

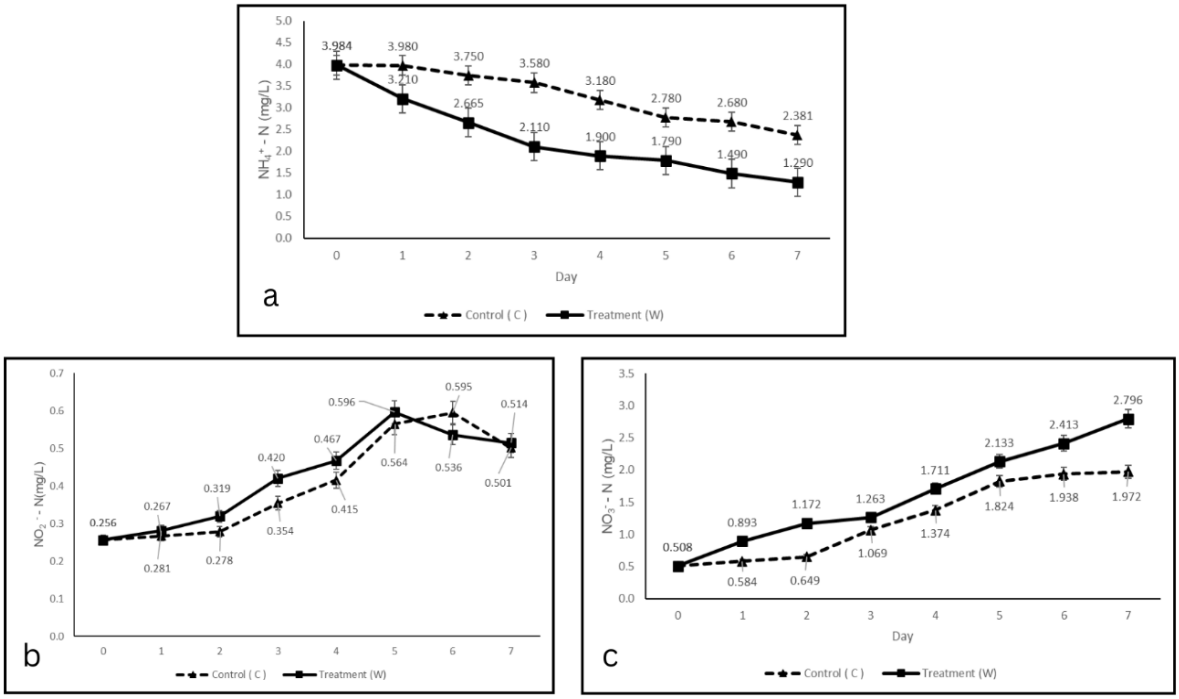

Figure 6. Water conditions: The nitrogenous compounds (a) NH_4^+—N, (b) NO_2—N, (c) NO_3—N

3.4 Principal Component Analysis

PCA was applied to integrate multivariate water quality parameters and temporal sampling points in order to elucidate the overall effects of Bacillus-based probiotic supplementation on the culture water system [37]. The analysis showed that the first two principal components explained 89.0% of the total variance, with PC1 accounting for 60.3% and PC2 contributing 28.7%, indicating that most of the system variability was effectively captured within a two-dimensional ordination space (Figure 7). The PCA ordination revealed a clear separation of sampling points along PC1, where baseline samples (C0 and W0) clustered on the negative axis, while the final treatment stage (W7) was distinctly positioned on the positive axis, suggesting a strong temporal shift in water quality conditions following probiotic application.

The separation along PC1 reflects a functional gradient primarily associated with nitrogen transformation processes, particularly the reduction of ammonium and the accumulation of nitrate observed in probiotic-treated systems [34]. This pattern indicates enhanced nitrification efficiency and improved coupling between ammonia-oxidizing and nitrite-oxidizing microbial communities, processes that are strongly facilitated by *Bacillus* spp. through organic matter degradation and stimulation of aerobic microbial metabolism [37]. In contrast, PC2 differentiated intermediate sampling points (W1 and C1) from both baseline and final stages, highlighting a transient ecological phase characterized by short-term microbial restructuring and physicochemical adjustments immediately following probiotic addition [37].

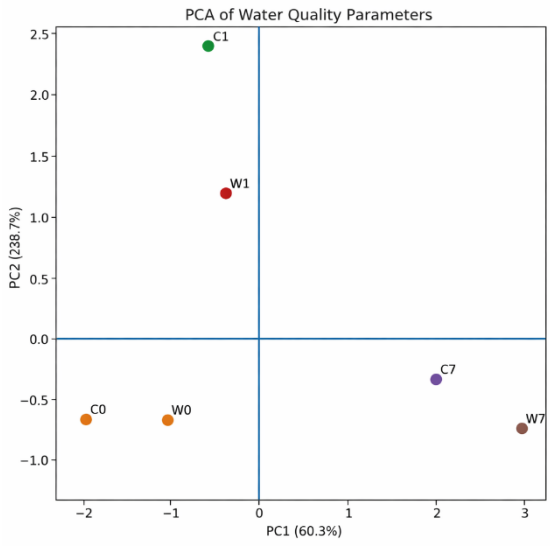

Figure 7. Principal Component Analysis (PCA) ordination of water quality parameters

The distinct positioning of W7 relative to earlier sampling points demonstrates that probiotic supplementation induced a shift toward a new and more stable ecological state rather than short-lived fluctuations [38]. This stabilization is consistent with the observed decrease in ammonium, controlled nitrite concentrations, increased nitrate levels, and elevated dissolved oxygen, all of which are indicative of improved water quality and system functionality [39]. Overall, the PCA confirms that Bacillus-based probiotic application acts as a major driver of multivariate water quality dynamics in *Litopenaeus vannamei* culture water, supporting enhanced nitrogen cycling efficiency while underscoring the importance of managing transitional phases during microbial succession [40].

3.5 Correlation analysis

Correlation analysis using Spearman's rank correlation Coefficient was conducted to evaluate the relationships among key water quality parameters, including temperature, dissolved oxygen (DO), pH, ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) [41]. The results revealed strong and structured interdependencies among these variables, indicating that water quality dynamics in the culture system were governed by tightly coupled physicochemical and biogeochemical processes rather than independent parameter fluctuations [42]. In particular, nitrogenous compounds and dissolved oxygen formed a coherent interaction network, highlighting the central role of aerobic microbial metabolism in regulating water quality [43].

A strong negative correlation was observed between DO and NH_4^+ ($\rho = -0.94$), while DO exhibited strong positive correlations with NO_2^- and NO_3^- ($\rho = 0.94$ for both) (Figure 8) [44]. This pattern provides compelling evidence of enhanced nitrification under well-oxygenated conditions, where ammonium is efficiently oxidized into nitrite and subsequently nitrate [45]. Such relationships are characteristic of active aerobic nitrogen cycling and are consistent with the observed reduction in ammonium and accumulation of nitrate in probiotic-treated systems [46]. The results support the functional role of Bacillus-based probiotics in stimulating oxidative microbial pathways, either directly through organic matter degradation or indirectly by promoting favorable conditions for nitrifying microbial consortia [47].

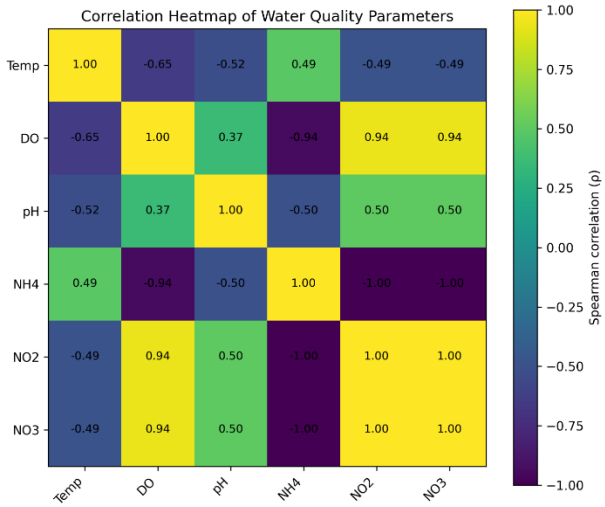

Figure 8. Spearman correlation heatmap of water quality parameters

Furthermore, the nitrogen species exhibited near-perfect correlations among themselves, with NH_4^+ showing strong negative correlations with both NO_2^- and NO_3^- ($\rho = -1.00$), and NO_2^- displaying a perfect positive correlation with NO_3^- ($\rho = 1.00$). These relationships suggest a tightly linked transformation cascade, where fluctuations in one nitrogen form are immediately reflected in others, indicative of a rapid and efficient nitrogen turnover system [48]. Moderate correlations between pH and nitrogen compounds further indicate that subtle chemical shifts may influence nitrogen speciation and microbial activity [49]. Overall, the correlation analysis reinforces the PCA findings and confirms that improvements in water quality were primarily driven by probiotic-mediated enhancement of aerobic nitrogen cycling and integrated system-level responses rather than isolated parameter changes [50].

4. DISCUSSION

The present study demonstrates that supplementation with a Bacillus-based probiotic at 1 ppm substantially altered microbial community structure and nitrogen dynamics in the culture water of *Litopenaeus vannamei*. However, it is important to acknowledge a key limitation of this work: microbial community data were only generated for the probiotic-treated group (W0–W7), while equivalent 16S rRNA profiling was not performed for the control group (C0–C7). Therefore, although the temporal changes observed in the W group are consistent with a probiotic-driven effect, the influence of natural time-dependent succession cannot be completely excluded. In this study, we thus interpret the observed community shifts primarily as responses to probiotic addition, while recognizing that part of the variation may also reflect intrinsic temporal dynamics of closed aquatic systems.

4.1 Probiotic-driven microbial succession

The activated probiotic was dominated by *Bacillus* spp., a group of Gram-positive, endospore-forming bacteria with high ecological resilience and the capacity to rapidly colonize aquatic environments [51, 52]. Together with *Paracoccus* and *Halomonas*, which are associated with denitrification and salinity-adapted metabolism [53], the inoculum represented a functionally complementary consortium. Following application, pronounced shifts in the water microbiome were observed from W0 to W7, characterized by increasing dominance of Pseudomonadota and enrichment of Gammaproteobacteria such as Vibrionaceae, Rhodobacteraceae, Flavobacteriaceae, and Alteromonadaceae. This pattern is typical of disturbed or nutrient-enriched aquatic systems, where fast-growing copiotrophic taxa outcompete slower-growing K-strategists [54].

At the genus level, succession toward *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, and *Marinobacter* indicates a transition to r-strategist heterotrophs capable of exploiting labile organic substrates and particulate matter [55]. Such restructuring suggests that probiotic application acted as an ecological driver that reshaped resource availability and microbial interactions, rather than simply increasing the abundance of Bacillus in the water column.

4.2 Pathogen suppression and emergence of opportunistic taxa

Heatmap and relative abundance analyses indicated a

temporal decline of *Vibrio* spp. after probiotic application, particularly by W7. This finding is consistent with previous reports that Bacillus can suppress *Vibrio* through antimicrobial compound production, quorum quenching, and competitive exclusion [56, 57]. Given that *Vibrio parahaemolyticus* and related species are major causative agents of AHPND and other vibrioses in shrimp aquaculture [58], this suppression represents a clear functional benefit of the probiotic.

At the same time, the enrichment of other opportunistic genera, including *Pseudoalteromonas*, *Alteromonas*, and potentially *Pseudomonas*, constitutes an important ecological risk signal. These taxa are well known as fast-growing opportunists and, under stressful or unbalanced conditions, some species may act as pathogens or secondary invaders in crustaceans and fish. Their proliferation implies that ecological niches vacated by suppressed *Vibrio* populations may be rapidly occupied by alternative competitors. In practical applications, such shifts should be monitored through routine microbial profiling and combined with management strategies such as maintaining high dissolved oxygen, controlling organic loading, and applying multi-strain probiotic consortia to prevent dominance of any single opportunistic group.

4.3 Enhancement of nitrogen cycling and water quality

Probiotic treatment significantly improved nitrogen transformation, as evidenced by the marked decrease in NH_4^+ and the concomitant increase in NO_3^- relative to the control. These trends, together with elevated dissolved oxygen, indicate stimulation of aerobic nitrification processes [59]. The involvement of *Bacillus*, *Paracoccus*, *Halomonas*, and other functionally relevant taxa likely promoted a more efficient coupling between ammonia oxidation and nitrate formation, thereby reducing the accumulation of toxic intermediates [60].

The suppression of ammonium and stabilization of nitrite are particularly important for shrimp health, as even sub-lethal concentrations of these compounds can impair growth and immune function. Thus, the observed improvements in water quality are consistent with the intended functional role of Bacillus-based probiotics as bioremediators and modulators of microbial nutrient cycling [61, 62].

4.4 Ecological trade-offs and management implications

Although probiotic supplementation enhanced nitrification and reduced *Vibrio* abundance, the concurrent enrichment of copiotrophic and opportunistic bacteria highlights that probiotic-driven restructuring is not ecologically neutral. Shifts toward Gammaproteobacteria-dominated assemblages may increase system productivity and organic matter turnover, but they also elevate the risk of opportunistic blooms if environmental conditions deteriorate [63]. This underlines the necessity of evaluating probiotics at the community and ecosystem levels rather than focusing solely on single-target pathogens.

From a management perspective, continuous monitoring of microbial community composition, coupled with water quality control and the use of multi-species probiotic formulations (e.g., combining *Bacillus* with Rhodobacteraceae or nitrifying consortia), may help stabilize microbial networks and reduce the likelihood of opportunistic pathogen emergence [64].

4.5 Limitations and future perspectives

The present study was conducted in a controlled, water-only system and lacked parallel microbial community analysis for the control group. Future research should incorporate full microbiome profiling of both control and treatment groups, extend observations to pond-scale systems, and include sediment and biofloc compartments. Such approaches will allow clearer separation of probiotic effects from natural temporal succession and provide a more comprehensive assessment of ecological risks and benefits under commercial farming conditions.

5. CONCLUSIONS

This study suggests that Bacillus-based probiotic application is associated with improved nitrogen dynamics and measurable shifts in microbial community structure in *Litopenaeus vannamei* culture water. Probiotic-treated systems showed a marked reduction in ammonium concentrations accompanied by nitrate accumulation and higher dissolved oxygen, indicating enhanced nitrification and overall water quality.

Microbial community analyses revealed pronounced temporal succession following probiotic addition, including reduced relative abundance of *Vibrio* alongside enrichment of fast-growing and opportunistic taxa. These patterns highlight that while Bacillus-based probiotics may support water quality improvement, they can also induce community-level restructuring with potential ecological trade-offs.

Given the short experimental duration, laboratory-scale conditions, and the absence of control-group microbial sequencing, the findings should be interpreted as correlative rather than causal. Overall, the results emphasize the importance of evaluating probiotics at the whole-community level and support the development of multi-strain formulations and field-scale validation for sustainable shrimp aquaculture.

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