



# Effects of Combined Probiotics *Bacillus* sp. BS-K1 and *Pseudomonas* sp. CN-B1 on Ammonium Removal and Disease Resistance in Freshwater Aquaculture

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## Abstract

In this study, two highly active functional bacterial strains, identified as *Bacillus* sp. BS-K1 and *Pseudomonas* sp. CN-B1, were isolated and applied in combination within a perch (*Maccullochella peelii*) recirculating aquaculture system (RAS) to improve water quality and enhance disease resistance against pathogenic bacteria. Experimental results revealed that the combined probiotics K1+B1 achieved inhibitory rates of 65.0% and 52.3% against *Staphylococcus aureus* and *Vibrio parahaemolyticus*, respectively. Remarkably, it exhibited removal efficiencies of 81.9%, 62.4%, and 68.8% for ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen under controlled conditions. Practical application in RAS demonstrated enhanced performance with ammonia and nitrite removal rates reaching 63.8% and 60.5%, respectively, significantly higher than those observed with individual strains. Metagenomic analysis showed altered microbial community structure after K1+B1 addition, with ACE and Chao1 indices increasing by 5%, indicating higher species richness. Notably, the relative abundance of functional genera involved in nitrogen cycling (*Novosphingobium* and *Aquabacterium*) carrying nitrifying and denitrifying capabilities increased significantly. Concurrently, pathogenic *Staphylococcus aureus* exhibited a dramatic reduction (>98%) compared to the control group. These results suggest that the K1+B1 consortium enhances water purification efficiency through strategic modulation of microbial community structure, promoting beneficial nitrogen-cycling populations while suppressing pathogenic microorganisms.

## Introduction

The recirculating aquaculture system (RAS), which integrates advanced concepts such as water treatment technology, high-density aquaculture, and intensive management, represents an environmentally friendly and sustainable model for aquaculture development. Pathogenic microorganisms and nitrogen pollution constitute two major challenges in high-density recirculating aquaculture system systems, posing significant threats to both aquatic organism health and environmental sustainability. Notably, pathogenic bacteria such as *Vibrio* spp., *Staphylococcus*

*aureus*, and *Escherichia coli* frequently induce disease outbreaks under intensive farming conditions. These pathogens invade hosts through epithelial wounds or mucosal surfaces, triggering clinical manifestations including ulcerative lesions, septicemia, and enteritis, which culminate in substantial mortality rates (Díaz et al., 2021). Concurrently, nitrogenous compounds derived from residual feed, fecal matter, and metabolic waste accumulate rapidly in high-density systems. Elevated nitrogen loads destabilize aquatic ecosystems by promoting harmful algal proliferation, hypoxic conditions, and progressive water quality degradation, ultimately impairing the growth performance of

cultured species and exerting detrimental ecological impacts (Hoang et al., 2022).

Current water purification strategies in aquaculture predominantly encompass physical, chemical, and biological approaches. While physical and chemical methods remain in use, they often entail substantial operational costs and carry risks of secondary contamination. In contrast, microbial-based technologies have emerged as a sustainable alternative in recent decades, owing to their cost-effectiveness and environmental compatibility. Notably, probiotics—recognized as sustainable alternatives to antibiotics—demonstrate multifaceted benefits, including pathogen suppression, water quality enhancement, and immunomodulatory effects on cultured species, thereby gaining prominence in aquaculture applications (Shao et al., 2021). For instance, He et al reported that low-dose supplementation with *Bacillus subtilis* C-3102 modulated the gut microbiota of tilapia (*Oreochromis niloticus*), inducing upregulation of innate immune cytokines (e.g., IL-1 $\beta$ , TNF- $\alpha$ ) and downregulation of stress-responsive HSP70, thereby promoting host-beneficial gene expression and microbiota homeostasis (He et al., 2013). Similarly, Nayak et al demonstrated that *Bacillus subtilis* strains B2 and B5 exhibited potent anti-*Vibrio* activity, achieving inhibition rates of >90% and >85%, respectively, after 120 hours of co-culture (Nayak S, 2012). Furthermore, nitrifying and denitrifying bacteria demonstrate significant capabilities in aquaculture applications. For instance, Shu et al. isolated two heterotrophic nitrification-aerobic denitrification (HN-AD) strains, designated S16 and DS5, from a freshwater aquaculture system. These strains exhibited inorganic nitrogen removal efficiencies ranging from 66.59% to 97.97% (S16) and 72.27% to 96.44% ('DS5'), respectively (Shu et al., 2022).

Despite their promise, the efficacy of single-strain probiotics is constrained by their limited functional diversity and poor adaptability to complex aquatic matrices, often resulting in suboptimal treatment performance. The synergistic enhancement of multi-species microbial consortia represents a pivotal trend in microbial ecology and applied microbiology. Through mechanisms such as metabolic complementation, quorum sensing, and spatial coordination, diverse microbial assemblages enable the realization of biological functions that are either unattainable by single-species cultures or achievable only with suboptimal efficiency. For example, in biological pest control, *Bacillus subtilis* secretes lipopeptide antibiotics, while *Pseudomonas spp.* produce siderophores to sequester iron ions essential for the growth of pathogenic bacteria. The co-application of these two taxa significantly potentiates the inhibitory activity against *Fusarium spp.* and *Ralstonia solanacearum*, thereby reducing the incidence of crop diseases (Raaijmakers et al., 2012). The synergistic effect of multiple strains through "division of labor and cooperation" to achieve functional enhancement

reflects the ecological wisdom of microbial communities and also provides a new strategy for solving the technical problems of complex and high-density aquaculture. Furthermore, the ecological impacts of probiotic consortia as water amendments—particularly their effects on microbial community dynamics and long-term ecosystem stability—remain insufficiently characterized, highlighting a critical research gap in sustainable aquaculture management.

Therefore, this study isolated two functionally distinct probiotic strains from natural environments: *Bacillus* sp. BS-K1, exhibiting antimicrobial properties, and *Pseudomonas* sp. CN-B1, demonstrating denitrification capabilities. These strains were formulated into a composite probiotic consortium, and their combined antimicrobial and nitrogen-removal efficiencies were systematically validated. Furthermore, the consortium was applied to a freshwater recirculating aquaculture system, where metagenomic approaches were employed to elucidate its impact on aquatic microbial community structure. This work advances the mechanistic understanding of multifunctional probiotic consortia in modulating aquatic microbiomes and provides novel insights into microbial-driven water purification strategies for high-density aquaculture systems.

## Materials and Methods

### Probiotic Cultivation and Combined Probiotics K1+B1 Preparation

*Bacillus* sp. BS-K1 (Genbank: PX908886) and *Pseudomonas* sp. CN-B1 (Genbank: PX908889) were isolated from the biofilter of a recirculating aquaculture system. For BS-K1, 1 g of biofilter carrier material was inoculated into 100 mL of sterile water and incubated for 2 h. Subsequently, serial dilutions ( $10^{-2}$ – $10^{-8}$ ) were spread onto LB solid medium. Single colonies were then selected for antagonism assays against pathogenic bacteria. A colony exhibiting strong antibacterial activity was further identified by 16S rRNA gene sequencing and designated as BS-K1. For the denitrifying bacterium, water samples were first enriched in nitrification medium. After gradient dilution ( $10^{-2}$ – $10^{-8}$ ), a group of nitrifying bacteria was isolated and inoculated into fresh nitrification medium to verify their performance. The strain demonstrating superior denitrification ability was selected and designated as CN-B1 for subsequent experiments.

Two probiotic strains— BS-K1 (antibacterial) and CN-B1 (denitrification capacity)—were isolated and utilized to prepare a composite probiotic consortium. Individual strains were inoculated into LB medium (pH 7.0–7.2) and incubated at 30°C with agitation (180 rpm) until reaching an optical density (OD<sub>600</sub>) of 1.0. Equal volumes of BS-K1 and CN-B1 seed cultures were then mixed at a 1:1 ratio to generate the combined probiotics K1+B1.

### Identification of 16S rDNA Strains

Selected single colonies were subjected to colony PCR employing primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTGTTACGA-3'), under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min; and a final extension at 72°C for 10 min. Purified PCR products were sequenced by Zhejiang Shangya Biotechnology Co., Ltd., China. BLAST analysis (GenBank<sup>1</sup>) of the obtained sequences identified 16 representative bacterial 16S rDNA sequences sharing high similarity. Phylogenetic relationships were assessed using MEGA7.0 to pinpoint the most closely related strains.

### Antibacterial Assay

The antibacterial activity of different concentrations of the combined probiotics K1+B1 against *Vibrio parahaemolyticus* and *Staphylococcus aureus* was evaluated using the agar well diffusion assay. Overnight cultures of *Staphylococcus aureus* and *Vibrio spp.* (OD<sub>600</sub> = 1.0) were individually mixed with sterilized LB agar (1:5 v/v) at 50°C. After solidification, 6-mm-diameter sterile filter discs were aseptically positioned at plate centers. The K1+B1 co-culture was serially diluted (10<sup>-1</sup>, 10<sup>-2</sup>) with sterile water. Inoculation was achieved by applying 100 µL aliquots of the K1+B1 consortium (1×10<sup>8</sup> CFU mL<sup>-1</sup>) onto discs using sterile applicators. Plates were incubated at 30°C for 48 hours, and the diameter of inhibition zones was measured. All experiments were conducted in triplicate to ensure reproducibility. The performance verification results were statistically analyzed by one-way ANOVA using IBM SPSS Statistics 27 software. Distinctions with P<0.05 (\*) or P<0.01 (\*\*) were deemed statistically noteworthy.

$$\% \text{ Inhibition rate} = \frac{D_1 - D_0}{D_1} \times 100$$

Where, D<sub>0</sub> = diameter of Blank Antimicrobial Susceptibility Disks (6 mm); D<sub>1</sub> = diameter of the inhibition zone.

### Denitrification Performance Evaluation

To evaluate denitrification capacity, a 1% (v/v) combined probiotics K1+B1 was transferred into nitrification medium (NM) supplemented with either NaNO<sub>2</sub>, KNO<sub>3</sub>, or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the sole nitrogen source, followed by incubation under identical conditions. Samples were collected at 12-hour intervals to determine the concentrations of NH<sub>4</sub><sup>+</sup>-N, NO<sub>2</sub><sup>-</sup>-N, and nitrate nitrogen (NO<sub>3</sub><sup>-</sup>-N), as well as the optical density at 600 nm (OD<sub>600</sub>). All experimental treatments were conducted in triplicate. The NM comprised 5.0g of sodium acetate, 0.235 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g of KH<sub>2</sub>PO<sub>4</sub>,

0.5 g of Na<sub>2</sub>HPO<sub>4</sub>, 0.4 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, and 2.00 mL of trace elements per liter (initial pH of 7).

### Effect of Adding Probiotic Complex on Water Quality of Aquaculture

This study was conducted in a freshwater recirculating aquaculture system rearing perch (*Maccullochella peelii*) as the target species (30°56'N, 120°53'E). The stocking density and the size of the breeding ponds are 50 KG/m<sup>3</sup> and 30 m<sup>3</sup> respectively. The seed cultures of composite consortium K1+B1, strain CN-B1, and strain BS-K1 were respectively transferred into fermenters for high-density fermentation. During scale-up cultivation, the inoculum-to-medium ratio in the fermenters was maintained at 1:100 (v/v). The fermentation parameters were controlled as follows: temperature 25-30°C, pH 7.0-7.2, agitation speed 180 rpm, and duration 24 h. The enriched bacterial inoculum was diluted 100-fold and added to the system at a dosage of 1 L/m<sup>3</sup>, with weekly supplementation at 7-day intervals." Concentrations of NH<sub>4</sub><sup>+</sup>-N and NO<sub>2</sub><sup>-</sup>-N in the water body were measured daily.

Seed medium: Glucose 5 g, K<sub>2</sub>HPO<sub>4</sub> 0.5 g, MgSO<sub>4</sub> 0.2 g; trace salt solution 2 mL, 1% (volume percentage) yeast extract solution, 1% (volume percentage) peptone solution, distilled water 1000 mL, pH 7.0 - 7.2. Among them: yeast extract solution concentration: 0.5 g/10 mL; peptone solution concentration: 1 g/10 mL.

### Effects of Adding Combined Probiotics K1+B1 on Microbial Community Structure in Cultured Water

Metagenomic analysis was employed to investigate the effects of probiotic composite consortium supplementation on microbial community structure in aquaculture water. Microbial samples were collected from water in both the experimental group amended with the combined probiotics K1+B1 and the control group without bacterial amendment by filtration through filter membrane (three replicates per group), and were then rapidly frozen in liquid nitrogen. Subsequent DNA extraction and quality control checks were performed, followed by sequencing of qualified DNA samples. This metagenomic analysis was performed on the Illumina NovaSeq platform utilizing a paired-end 150-base (PE150) sequencing strategy for libraries with an average insert size of approximately 350 bp. Raw sequencing data were first subjected to stringent quality control using the fastp software. The filtering criteria included the removal of read pairs containing adapter sequences, read pairs where over 50% of bases in either read had a quality score (Q) ≤5, and read pairs with an N content exceeding 10% in either read, resulting in high-quality clean data.

Subsequent bioinformatic analyses followed a standardized pipeline. De novo assembly of the quality-filtered reads was conducted using MEGAHIT with the --

presets meta-large (--end-to-end, --sensitive, -l 200, -X 400) parameter. Open reading frames (ORFs) were predicted from the assembled contigs (scaffolds) with a length of  $\geq 500$  bp using MetaGeneMark, and predicted ORFs shorter than 100 nucleotides were filtered out. A non-redundant gene catalog was constructed by clustering the predicted genes with CD-HIT using parameters -c 0.95 and -aS 0.9. Gene abundance was quantified by aligning the clean reads back to this gene catalog using Bowtie2, and genes supported by two or fewer reads in a given sample were subsequently filtered out to eliminate low-abundance artifacts.

DIAMOND software is used for alignment of unigenes sequences with Micro\_NR database, which includes sequences from bacteria, fungi, archaea, and viruses extracted from NCBI's NR database. Since each sequence may have multiple alignment results, LCA algorithm (applied to systematic taxonomy of MEGAN software is adopted to determine the species annotation information of the sequence (Huson et al., 2011). Out of the results of LCA annotation and gene abundance table, the abundance of each sample at each taxonomy (kingdom, phylum, class, order, family, genus, or species) and the corresponding gene abundance tables are acquired. The abundance of a species in a sample is equal to the sum of the abundance of those genes annotated as that species (Karlsson et al., 2012) **Hata! B aşvuru kaynağı bulunamadı..** The number of genes of a species in a sample is equal to the number of genes whose abundance is non-zero among the genes annotated as that species.

### Data Analysis

On the basis of the abundance tables at each taxonomy level, Krona analysis, relative abundance overview, and abundance clustering heatmap are performed, combined with PCA (R ade4 package), PCoA, and NMDS (R vegan package) analysis of dimension reduction. Anosim analysis (R vegan package) is used to test the differences between groups. MetaGenomeSeq and LEfSe analysis are used to search for species differences between groups. MetaGenomeSeq analysis is used to perform permutation test between groups on each taxonomy level and get a p-value. Distinctions with

$P < 0.05$  (\*) or  $P < 0.01$  (\*\*) were deemed statistically noteworthy. The experiment was carried out with three replicates under identical experimental conditions.

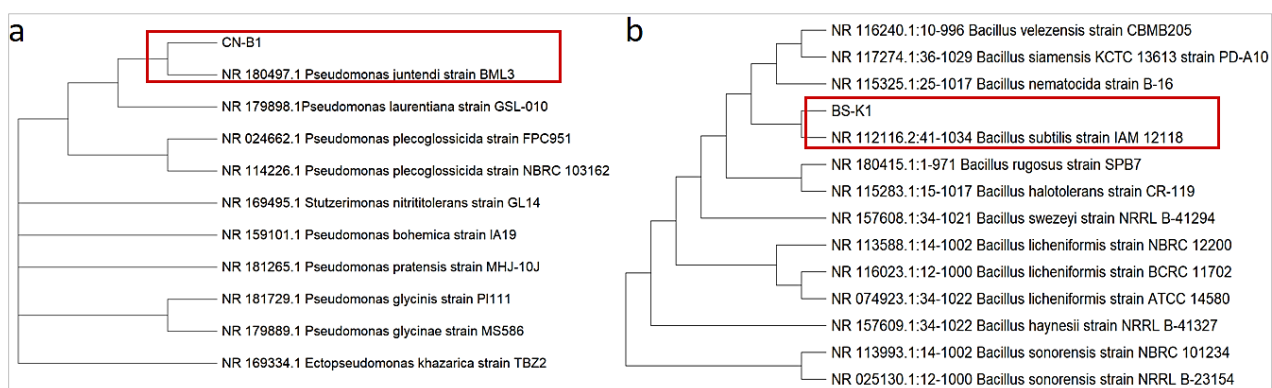
## Results and Discussion

### Identification of Strains

Through high-throughput screening technology, a strain with excellent denitrification capability, designated as *Pseudomonas* sp. CN-B1, was isolated and screened. BLAST analysis of its 16S rDNA gene sequence indicated sequence identity (approximately 99.5%) with *Pseudomonas juntendi*. Additionally, a strain with notable antibacterial activity, named *Bacillus* BS-K1, was isolated. BLAST analysis of its 16S rDNA gene sequence revealed sequence identity (approximately 99.5%) with *Bacillus subtilis*. The phylogenetic trees of these two strains are illustrated in Figure 1.

### Verification of Antibacterial Properties of Combined Probiotics K1+B1

*Staphylococcus aureus* and *Vibrio* spp. constitute prevalent pathogenic bacteria in aquaculture water systems (Wang et al., 2021; Yaylacı, 2021). The antibacterial of the combined probiotics K1+B1 against target pathogens was evaluated using the agar well diffusion assay. As shown in Figure 2a and 2b, K1+B1 exhibited inhibitory effects against *Staphylococcus aureus* and *Vibrio parahaemolyticus*. As demonstrated in Figure 2c, the undiluted K1+B1 consortium exhibited potent inhibition against *S. aureus*, generating an inhibition zone of  $17.4 \pm 2.0$  mm (65.0% inhibition rate). 10-fold diluted suspensions maintained moderate activity ( $10.2 \pm 0.8$  mm inhibition zone, 41.2% inhibition rate), while 100-fold dilutions showed markedly reduced effectiveness ( $7.3 \pm 0.5$  mm, 16.8% inhibition rate). As shown in Figure 2d, the undiluted K1+B1 consortium exhibited potent inhibition against *Vibrio parahaemolyticus* generating an inhibition zone of  $12.6 \pm 1.2$  mm (52.3% inhibition rate), decreasing to  $8.9 \pm 1.0$  mm (32.8% inhibition) at 10-fold dilution. Notably, 100-fold diluted suspensions exhibited negligible anti-*Vibrio* activity (<5% inhibition).



**Figure 1.** Phylogenetic trees of strains (a) Phylogenetic tree of CN-B1; (b) Phylogenetic tree of BS-K1.

These results indicate that the K1+B1 consortium displays superior antimicrobial performance against *S. aureus* compared to *Vibrio parahaemolyticus*, while maintaining therapeutically relevant inhibition against both pathogens at optimal concentrations.

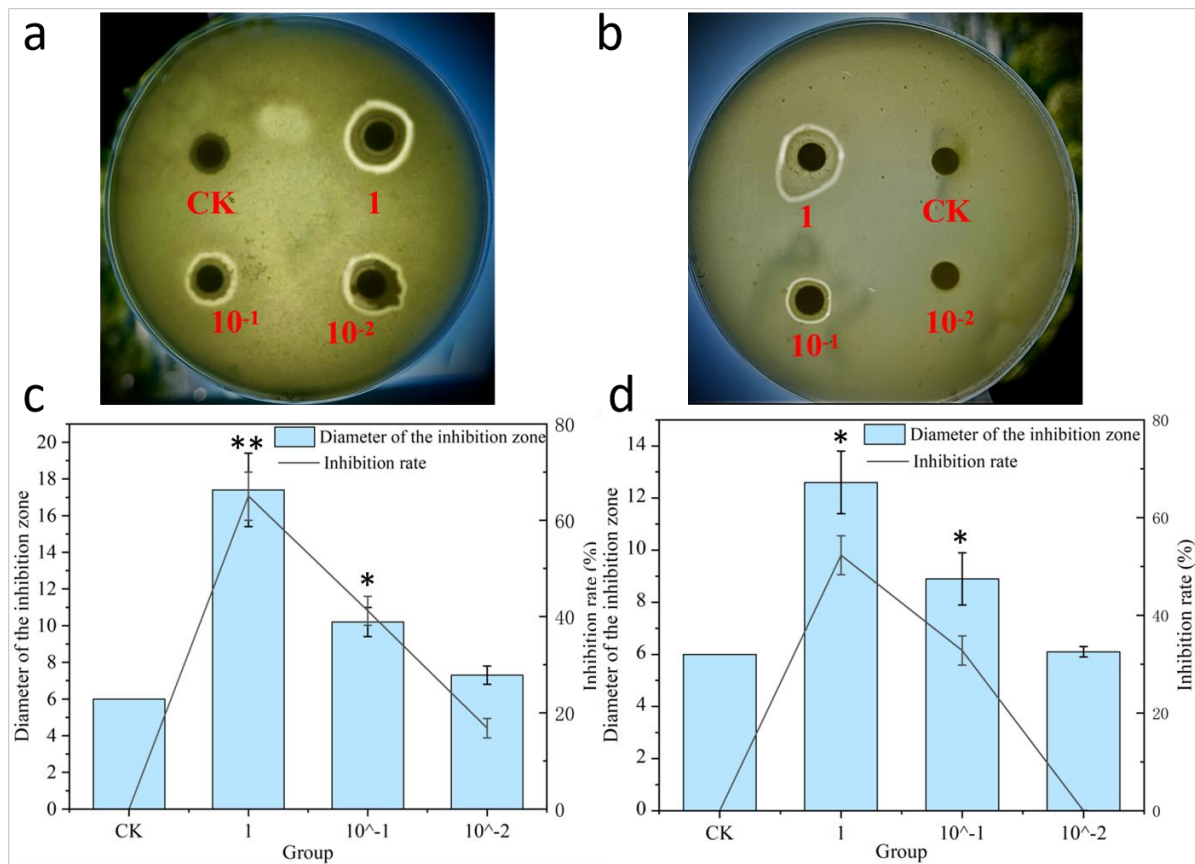
**Verification of Denitrification Performance of Combined Probiotics K1+B1**

NH<sub>4</sub><sup>+</sup>-N, NO<sub>2</sub><sup>-</sup>-N, and NO<sub>3</sub><sup>-</sup>-N are all critical factors affecting water quality in aquatic environments. This experiment investigated the denitrification efficacy of the combined probiotics K1+B1 under different nitrogen sources. As illustrated in Figure 3a, when NH<sub>4</sub><sup>+</sup>-N was used as the nitrogen source, the OD<sub>600</sub> value significantly increased from 0 h to 12 h, reaching 0.529, and subsequently continued to rise to 0.768 by 36 h, before beginning to decline between 36 h and 48 h. When NO<sub>2</sub><sup>-</sup>-N and NO<sub>3</sub><sup>-</sup>-N were used as nitrogen sources, the growth trends were similar to those in the ammonium nitrogen group, with rapid growth from 0-12 h and peak OD<sub>600</sub> values at 24 h and 36 h, reaching 0.695 and 0.701, respectively. At all time points, when nitrate nitrogen was used as the nitrogen source, the OD<sub>600</sub> values were generally higher than those with ammonium nitrogen and nitrite nitrogen, indicating the most vigorous growth of the strains under nitrate nitrogen conditions.

The denitrification effects of the K1+B1 are shown in Figure 3b. When NH<sub>4</sub><sup>+</sup>-N was used as the nitrogen source, the initial concentration in the medium was 18.2 mg/L, which gradually decreased over time. From 0 h to 12 h, the concentration decreased from 18.2 mg/L to 3.3 mg/L, and despite slight fluctuations, it maintained a general downward trend, reaching 3.52 mg/L at 48 h. The maximum removal rate of ammonium nitrogen during this period was 81.9%. The TN concentration was 22.1 mg/L, which also gradually decreased over time. From 0 h to 12 h, the concentration significantly decreased to 6.3 mg/L, and at 48 h, it was 6.53 mg/L, with a maximum TN removal rate of 73.2%. Furthermore, no accumulation of NO<sub>2</sub><sup>-</sup>-N or NO<sub>3</sub><sup>-</sup>-N was observed based on the data.

When NO<sub>3</sub><sup>-</sup>-N was used as the nitrogen source (Figure 3d), the initial TN concentration was 23.1 mg/L, which subsequently decreased. It dropped to 9.8 mg/L at 12 h, further to 8.9 mg/L at 24 h, and then the rate of decrease slowed, reaching 8.3 mg/L at 48 h. The initial nitrate nitrogen concentration was 16.8 mg/L, which decreased to 6.1 mg/L at 12 h and to 6.3 mg/L at 48 h. The TN removal rate was 64.1%, and the nitrate nitrogen removal rate was 68.8%. No accumulation of NH<sub>4</sub><sup>+</sup>-N or NO<sub>2</sub><sup>-</sup>-N was observed based on the data.

When NO<sub>2</sub><sup>-</sup>-N was used as the nitrogen source (Figure 3c), the initial TN concentration was 22.3 mg/L, which subsequently decreased. It dropped to 12.3 mg/L



**Figure 2.** Antagonistic interactions between strains.(a) Antagonistic activity of the K1+B1 probiotic consortium against *Staphylococcus aureus*. (b) Antagonistic activity of the K1+B1 probiotic consortium against *Vibrio parahaemolyticus*. (c) The inhibition rate of K1+B1 against *Staphylococcus aureus*. (d) The inhibition rate of K1+B1 against *Vibrio parahaemolyticus*.

at 12 h, further to 10.1 mg/L at 24 h, and then the rate of decrease slowed, reaching 9.3 mg/L at 48 h. The initial nitrite nitrogen concentration was 18.7 mg/L, which decreased to 8.7 mg/L at 12 h and to 7.8 mg/L at 48 h. The maximum TN removal rate during this period was 58.3%, and the maximum nitrite nitrogen removal rate was 62.4%. No accumulation of  $\text{NH}_4^+\text{-N}$  or  $\text{NO}_3^-\text{-N}$  was observed based on the data. Based on these results, the K1+B1 demonstrates efficient removal of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  without causing the accumulation of harmful substances.

**Impact of Combined Probiotics K1+B1 on Water Quality in Aquaculture Systems**

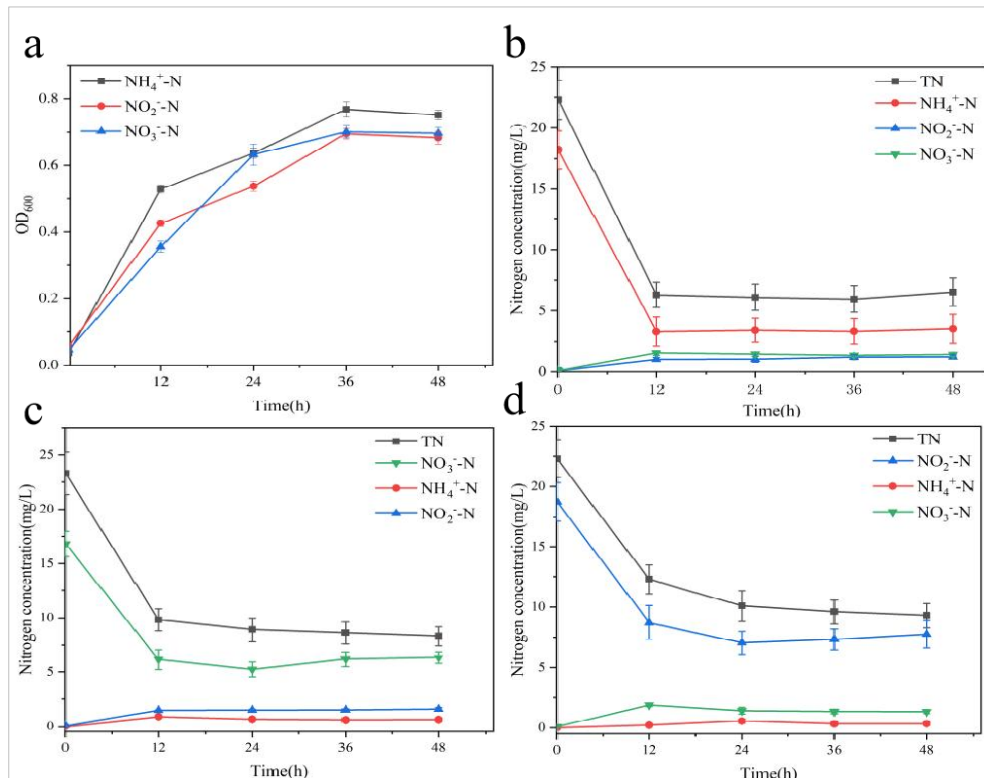
Ammonia nitrogen and nitrite nitrogen are critical indicators that require close attention during aquaculture processes, as they significantly influence aquaculture outcomes. This experiment compared the changes in water quality in a recirculating aquaculture system after the addition of the composite bacterial agent K1+B1, single *Pseudomonas* sp. CN-B1, and single *Bacillus* sp. BS-K1 agents. The results are depicted in Figure 4. The results (Figure 4a) indicate that during the 7-day period before the addition of any bacterial agents, the initial ammonia nitrogen concentration in the recirculating aquaculture system ranged from 2.0 to 2.4 mg/L. After the addition of the bacterial agents, a notable downward trend in ammonia nitrogen concentration was observed across all three experimental groups. Specifically, the concentration stabilized at approximately 1.0 mg/L in the K1+B1 group,

around 1.5 mg/L in the CN-B1 group, and approximately 1.8 mg/L in the BS-K1 group. Among these, the K1+B1 group exhibited the highest removal rate of 63.8%, followed by the CN-B1 group with a removal rate of 32.1%, which, although lower than that of the K1+B1 group, still demonstrated a certain level of removal efficacy. The BS-K1 group had the lowest removal rate at 27.3%, indicating a relatively poor performance in ammonia nitrogen removal.

As shown in Figure 4b, the nitrite nitrogen concentration in the water of the three groups before the addition of bacterial agents ranged from 1.3 to 1.7 mg/L. The data revealed that the nitrite nitrogen concentration stabilized at approximately 0.6 mg/L in the BS-K1 group, around 0.9 mg/L in the CN-B1 group, and approximately 1.20 mg/L in the BS-K1 group. The removal rates of nitrite nitrogen concentration for the three groups were 60.5%, 40.1%, and 25.2%, respectively. In summary, within the complex aquatic environment of aquaculture systems, the efficacy of individual bacterial strains tends to decline, whereas the combined probiotics K1+B1 demonstrates superior adaptability and enhanced removal efficiency for both ammonia nitrogen and nitrite.

**Impact of Combined Probiotics K1+B1 on Water Microbial Community Diversity**

In this study, microbial sequences from four categories—Bacteria, Fungi, Archaea, and Viruses—were extracted from the database for microbial analysis. For each sequence alignment result, those with an e-



**Figure 3.** Denitrification performance of the K1+B1. (a) OD<sub>600</sub> values of K1+B1 under different nitrogen sources; (b)  $\text{NH}_4^+\text{-N}$  removal efficiency; (c)  $\text{NO}_3^-\text{-N}$  removal efficiency; (d)  $\text{NO}_2^-\text{-N}$  removal efficiency.

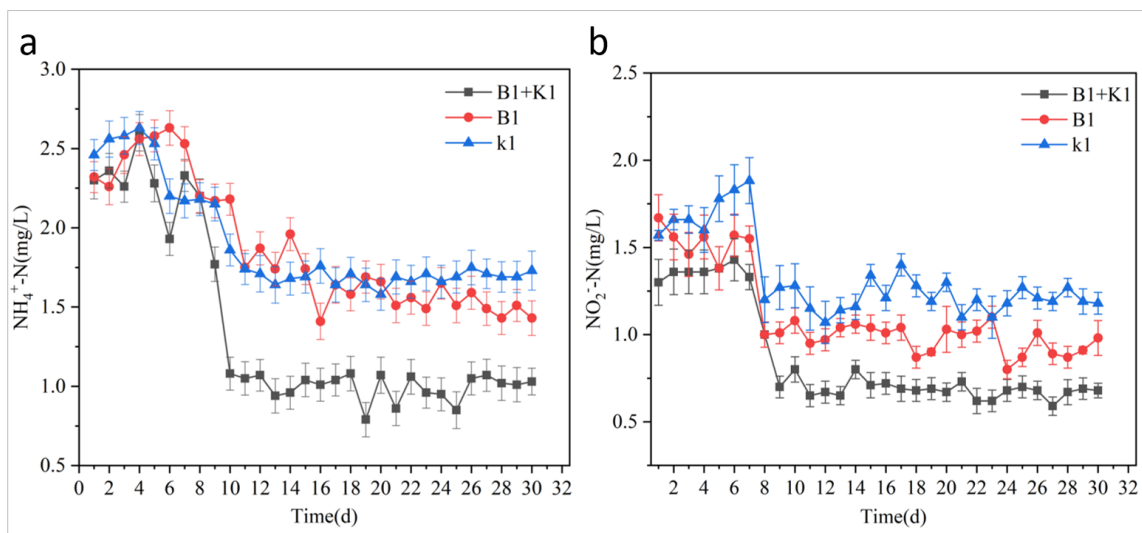
value  $\leq 10^{-5}$  were selected. Given that each sequence might have multiple alignment results, the Lowest Common Ancestor (LCA) algorithm was employed to determine the species annotation information for the sequence, ensuring the rationality of the microbial sequence analysis. The results are depicted in Figure 5.

The results indicate significant differences in microbial community diversity between the K1+B1 group and the CK group ( $P < 0.05$ ). As shown in Figures 5a and 5b, the K1+B1 group, which received the composite bacterial agent, exhibited an ACE index of 16,260 and a Chao1 index of 16,328. In contrast, the CK group, which did not receive the composite bacterial agent, had an ACE index of 15,592 and a Chao1 index of 15,693. Both the ACE and Chao1 indices are used to assess species richness in a sample, with higher values indicating a greater number of species or operational taxonomic units (OTUs) (Hassan et al., 2022). Consequently, the K1+B1 group demonstrated significantly higher species richness compared to the CK group. Overall, the addition of the probiotic composite bacterial agent led to noticeable changes in microbial diversity within the water body.

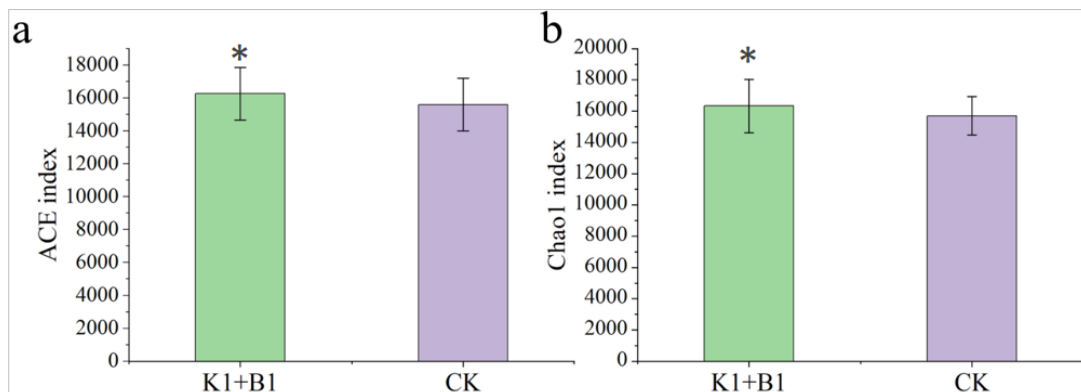
### Impact of Adding Combined Probiotics K1+B1 on the Microbial Community Structure in Aquaculture Water

At the Kingdom level (Figure 6a), both the K1+B1 group and the CK group exhibited the presence of microorganisms such as Bacteria, Fungi, Archaea, and Viruses. The data from both groups indicated that bacteria accounted for 80.53% in the K1+B1 group and 74.53% in the CK group. Notably, the viral abundance in the CK group (5.86%) was significantly higher than that in the K1+B1 group (3.42%). Archaea and Eukaryota each accounted for less than 0.2% in both groups, suggesting that bacteria constitute a crucial component of the indigenous microbial population and serve as primary drivers in biogeochemical processes such as carbon and nitrogen cycling (Olmos et al., 2019; Zhang et al., 2022). The higher bacterial abundance in the K1+B1 group may reflect more active carbon and nitrogen cycling.

At the Phylum level (Figure 6b), the dominant phyla in both the K1+B1 and CK groups included *Proteobacteria*, *Podoviridae*, *Bacillota*, *Bacteroidota*, and *Actinobacteriota*. Among these, *Proteobacteria* had



**Figure 4.** Water quality dynamics in the recirculating aquaculture system (RAS). (a) Temporal variation of  $\text{NH}_4^+-\text{N}$  concentrations; (b) Temporal variation of  $\text{NO}_2^--\text{N}$  concentrations.

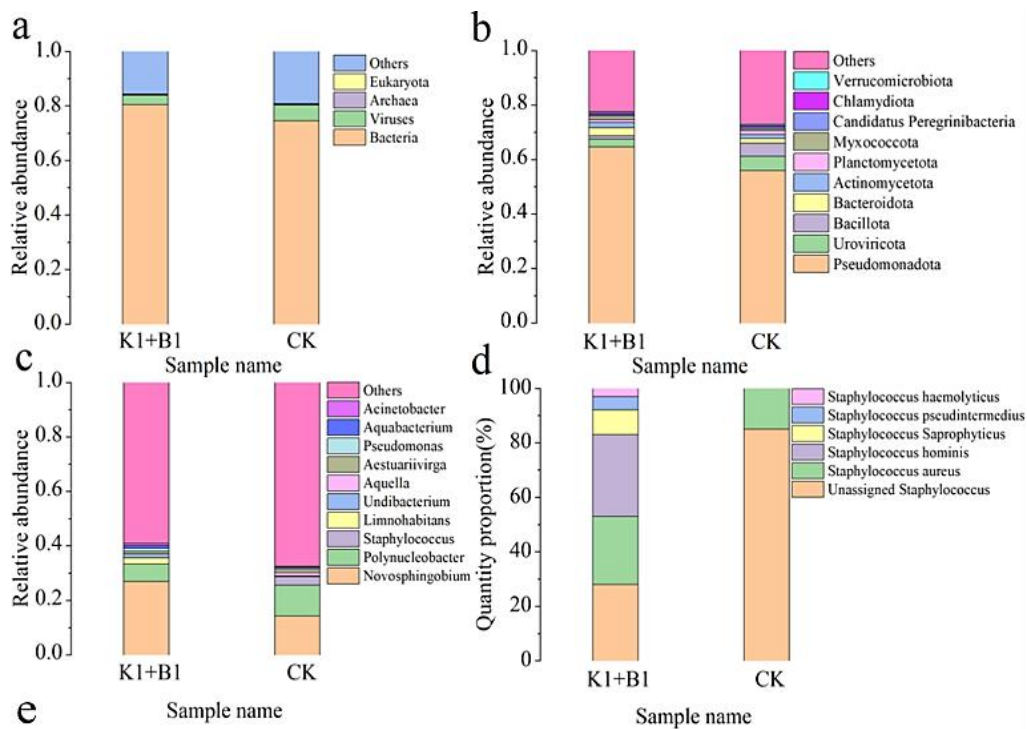


**Figure 5.** Microbial community diversity analysis. (a) ACE index of K1+B1 and CK ;(b) Chao1 index of K1+B1 and CK.

the highest relative abundance in both groups, with values of 0.65 and 0.56, respectively, followed by *Bacteroidota* with relative abundances of 0.03 and 0.018. Previous studies have identified *Proteobacteria* and *Bacteroidota* as major heterotrophic bacteria responsible for organic matter removal and critical contributors to nitrogen and phosphorus cycling, with nitrification and denitrification capabilities that are vital for maintaining water carbon and nitrogen cycles (Lerner et al., 2019; Soni et al., 2021).

At the Genus level (Figure 6c), the top genera in both the K1+B1 and CK groups were *Novosphingobium*, *Polynucleobacter*, *Staphylococcus*, *Limnohabitans*, *Aquabacterium*, and *Acinetobacter*. The relative abundance of *Novosphingobium* was 0.26 in the K1+B1

group, higher than the 0.14 in the CK group. Previous research has shown that *Novosphingobium* species possess the ability to degrade organic pollutants, demonstrating potential applications in environmental pollution control and ecological restoration (Li et al., 2024; Naiel et al., 2022). Additionally, the relative abundance of *Aquabacterium* was 0.01 in the K1+B1 group, higher than the 0.002 in the CK group. *Aquabacterium* species are widely distributed in various aquatic environments, including freshwater, seawater, and sediments, where they play important ecological roles in biogeochemical processes such as carbon and nitrogen cycling, crucial for maintaining carbon-nitrogen balance in water bodies (Kathia et al., 2018). Furthermore, the abundance of certain pathogenic



**Figure 6** Microbial structure analysis. (a) Species structure composition at the Kingdom level; (b) Species structure composition at the Phylum level; (c) Species structure composition at the Genus level; (d) Composition of *Staphylococcus* in K1+B1 and CK. (e) The growth condition of some aquaculture animals.

genera, such as *Staphylococcus*, was notably reduced in the K1+B1 group. *Staphylococcus aureus*, a common pathogen in the *Staphylococcus* genus, can cause various infections, including skin infections, pneumonia, and septicemia (Li et al., 2022). The relative abundance of *Staphylococcus* in the K1+B1 group was 0.00024, compared to 0.032 in the CK group. Specifically, *Staphylococcus aureus* accounted for 25.0% of the *Staphylococcus* genus in the K1+B1 group and 15% in the CK group (Figure 6d), indicating a 98.7% reduction in relative abundance in the K1+B1 group compared to the CK group. The growth status of cultured organisms in both experimental groups was observed (Figure 6e). The results revealed that although skin ulcerations were observed in both groups, the CK group exhibited a significantly higher prevalence of ulcerated fish with more severe symptoms compared with the K1+B1 group. In contrast, the group supplemented with the K1+B1 showed a lower incidence of dermatological disorders, demonstrating both reduced occurrence rates and attenuated symptom severity.

Therefore, the addition of the composite bacterial agent significantly influences the microbial community structure in water bodies, not only by increasing bacteria involved in nitrogen and carbon metabolic processes but also by effectively reducing the number of pathogenic microorganisms, fostering a favorable microbial community structure, and further improving water quality.

## Conclusions

In conclusion, our findings indicate that the combined probiotics K1+B1 exhibits dual functionalities of bacteriostasis and nitrogen removal. The probiotic mixture showed significant antibacterial activity against *Staphylococcus aureus* and *Vibrio parahaemolyticus*, while achieving removal rates of 63.8% for ammonia nitrogen and 60.5% for nitrite in aquaculture water. Metagenomic analysis revealed substantial alterations in microbial community structure following K1+B1 administration. Notably, we observed a marked increase in the abundance of bacterial taxa associated with nitrification, denitrification, and nitrogen-carbon cycling processes. Concurrently, the relative abundance of the pathogenic *S. aureus* showed significant reduction. These results indicate that the probiotic mixture enhances water purification efficacy by reshaping microbial communities towards beneficial configurations. This study provides novel insights into the mechanistic impacts of combined probiotics on aquatic ecosystems and proposes an effective eco-friendly approach for intensive aquaculture management.

## Ethical Statement

After being reviewed by the Experimental Animal Ethics Committee of Zhejiang A&F University (EAEC-

ZAFU), it is determined that the experimental animals, research content, and research plan involved in this project comply with the requirements of experimental animal ethics and animal welfare.

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## Author Contribution

**Y.T.** Methodology, Formal analysis, Data curation, Investigation, Writing – original draft; **T.X.** Resources, Formal analysis, Writing – review & editing, Investigation; **W.X.** Methodology, Formal analysis, Investigation; **Z.F.** Methodology, Data curation, Formal analysis, Investigation; **L.X.** Methodology, Data curation, Formal analysis; **P.J.** Conceptualization, Supervision, Methodology; **B.P.** Investigation, Formal analysis, Data curation; **Z.Z.** Project administration, Conceptualization, Writing – review & editing.

## Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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