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Multifunctional PNSB as a Live Feed Enhancer: Improving Artemia Performance and Disease Resistance

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ABSTRACT

The application of *Artemia* as a live feed in aquaculture is often limited by the high cost and variable quality of commercial cysts. This study explores the potential of two mangrove-associated purple non-sulfur bacteria (PNSB) strains - *Rhodopseudomonas palustris* AZR1 and *Rhodopseudomonas faecalis* AZW1 - as probiotic feed supplements to enhance the nutritional and health status of Artemia. The *Artemia*-fed treatments (AFT) received yeast and *Artemia* twice daily at 3.0 x 10^7 cells/mL for 14 days, whereas the PKC Nutri+ treatment was maintained at an OD_{600} of 0.3. Flavonoid contents in the PNSB strains and *Artemia*-fed treatments (AFT) were measured by spectrophotometry and HPLC. Nutritional contents were assessed using proximate analysis, and fatty acid contents were quantified via GC-MS. Twenty AFT were challenge with 1×10^8 CFU/mL of *Vibrio campbellii* and survival were observed after 72hr. Strain characterization revealed that *R. palustris* AZR1 exhibited superior carotenoid production, with significantly higher astaxanthin (0.324 µg/mL) and β-carotene (0.228 µg/mL) levels compared to *R. faecalis* AZW1 (0.254 and 0.160 µg/mL, respectively; $p < 0.05$). Additionally, *R. palustris* AZR1 showed a significantly greater total lipid content (7.77%) than *R. faecalis* AZW1 (6.76%; $p < 0.05$), despite overall comparable nutritional profiles. When used as a live feed, *R. palustris* AZR1 significantly enhanced *Artemia* growth, with differences becoming statistically significant from Day 4 onward ($p < 0.05$). Under gnotobiotic conditions, *Artemia* fed *R. palustris* AZR1 reached a mean length of 9.6 ± 0.32 mm by Day 14, outperforming those fed PKC Nutri+ (7.6 ± 0.43 mm) and yeast (7.5 ± 0.25 mm). A similar growth advantage was observed under hatchery conditions, where *R. palustris* AZR1-fed *Artemia* attained the highest length (10.1 ± 0.08 mm), surpassing both PKC Nutri+ (9.7 ± 0.55 mm) and yeast (8.5 ± 0.36 mm). Moreover, *Artemia* supplemented with *R. palustris* AZR1 demonstrated enhanced resistance to *Vibrio campbellii* challenge, which correlated with the upregulation of key immune-related genes, including Hsp70, Hsp90, and proPO. Nutritional analysis confirmed effective transfer of beneficial biomolecules, including carotenoids and lipids, from the PNSB to Artemia. While PKC Nutri+ was richer in polyunsaturated fatty acids (PUFAs), *R. palustris* AZR1 offers a safer, toxin-free alternative. In conclusion, this study highlights the potential of the mangrove-derived *R. palustris* AZR1 as a sustainable, multifunctional single-cell protein source with strong probiotic properties, capable of improving Artemia growth, immune competence, and nutritional quality in aquaculture systems.

Keywords: Artemia; *Rhodopseudomonas palustris;* probiotics; single-cell protein; nutritional quality; carotenoids; immune response

1. Introduction

Aquaculture is vital for global food security, with shrimp production being a key commercial sector. A major hurdle in early larval rearing is the reliance on live feeds, predominantly *Artemia* nauplii. Despite their favorable nutritional profile and high digestibility, *Artemia* use faces significant constraints, including high cost, inconsistent supply, variable quality, and, critically, biosecurity risks from potential pathogen vectors like *Vibrio* species [1].

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To address these issues, Purple Non-Sulfur Bacteria (PNSB) have emerged as a promising, sustainable single-cell protein alternative. PNSB offer a rich composition of proteins, lipids, essential fatty acids (PUFAs), and vitamins [2]. Beyond their superior nutrition, PNSB also function as probiotics, inhibiting pathogens (e.g., *Vibrio* spp.) and enhancing host digestion and water quality [3].

The selection of mangrove-derived PNSB strains based on their unique adaptation to extreme environmental conditions. Originating from mangrove ecosystems, these microorganisms naturally possess high tolerance to severe fluctuations in salinity, oxygen levels, and pH, ensuring exceptional stability and survival within challenging marine or brackish aquaculture waters. This inherent hardiness allows them to maintain superior performance and nutrient recycling efficiency, effectively consuming harmful nitrogenous wastes even under stressful culture conditions [4].

While PNSB supplementation improves growth performance and disease resistance in aquatic species, the molecular mechanisms underlying their interaction with *Artemia*, specifically concerning growth and immune gene expression, are poorly defined. This study investigates the potential of mangrove-derived PNSB strains to enhance *Artemia* by assessing their nutritional profile, effect on performance, disease resistance, and immune-related gene expression. We focus on the impact of selected strains, particularly *Rhodopseudomonas palustris* AZR1, on *Artemia* under controlled and hatchery conditions.

2. Methodology

2.1 PNSB Characterization

2.1.1 Growth and biomass production

Rhodopseudomonas palustris AZR1 (AZR1) and *R. faecalis* AZW1 (AZW1) was cultivated in triplicate in 112 broths under anaerobic, illuminated conditions (30 ± 2° C, PPFD:33.7 µmol.m^{-2}. s^{-1}) for 15 days. Growth was monitored daily by Optical Density (OD660) and Dry Cell Weight (DCW) [5].

2.1.2 Nutritional and carotenoid analysis

Lyophilized PNSB biomass (14-day culture) was subjected to proximate analysis (Moisture, Ash, Crude Protein, Crude Lipid, Crude Fibre) following AOAC (2019) [6] guidelines. Fatty Acid Methyl Ester (FAME) analysis was performed via direct transesterification and Gas Chromatography (GC-FID) to determine Saturated (SFA), Monounsaturated (MUFA), and Polyunsaturated Fatty Acid (PUFA) profiles. Total carotenoid content was determined spectrophotometrically (A_{480}), and qualitative/quantitative profiling was performed using High-Performance Liquid Chromatography (HPLC-PDA) with β-carotene and astaxanthin standards.

2.3 Artemia Bioassay

2.3.1 Hatching and experimental setup

Artemia nauplii (Stage II) were hatched from disinfected cysts (INVE Aquaculture). The main feeding trial was conducted over 14 days in 1-tonne tanks (500 L aerated seawater) with three replicate tanks per treatment, stocked at 200,000 nauplii. Three diets were tested: Yeast control (*Saccharomyces cerevisiae*), PKC Nutri+ (fermented palm kernel cake extract), and the PNSB diet (*R. palustris* AZR1). All feeds were standardized by cell density and administered twice daily.

2.1.3 Growth, survival, and water quality

Growth performance was monitored daily via total length measurements (mm) and survival was recorded. Water quality (T, S, pH, DO, NH_4^+) was monitored daily using calibrated meters and commercial test kits.

2.1.4 Gnotobiotic assay and disease challenge

An axenic (gnotobiotic) Artemia experiment was set up using decapsulated cysts hatched and cultured under sterile conditions [7] to control microbial exposure. After 14 days of feeding the experimental diets, disease resistance was assessed by exposing adult Artemia to a virulent *Vibrio campbellii* ($1x10^8$ CFU/mL) challenge. Cumulative mortality was recorded over 72 hours.

2.1.5 Nutritional Transfer and Photopigment Analysis

Artemia biomass from all treatments (Day 14) underwent proximate analysis and FAME analysis to assess nutritional transfer. Carotenoid uptake was qualitatively screened by UV-Vis spectrophotometry (300–600 nm) and quantified via HPLC [8].

2.1.6 Immune-Related Gene Expression (qPCR)

Immune and stress-related gene expression Hsp70, Hsp90, proPO) was analyzed via quantitative Real-Time PCR (qPCR) on Artemia collected on Days 1, 7, and 14. Total RNA was extracted, reverse-transcribed to cDNA, and qPCR was performed using a SYBR No-ROX system with β-actin and α-tubulin as reference genes. Relative expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method [9].

2.2 Statistical Analysis

All data were analyzed using SPSS (v22.0). Comparisons between two groups were done using the independent samples T-test, while comparisons across multiple groups utilized one-way ANOVA followed by Tukey's multiple range test ($p < 0.05$).

3. Results and Discussions

3.1 PNSB Nutritional Characterization

AZR1 demonstrated superior growth kinetics and carotenogenic activity compared to AZW1. AZR1 achieved a significantly higher maximum DCW (4.93 g/L vs. 3.90 g/L; $p < 0.05$) and greater total carotenoid content (10.16 mg/g vs. 7.68 mg/g; $p < 0.05$). HPLC analysis confirmed that AZR1 produced higher concentrations of key pigments, including astaxanthin (0.324 µg/mL) and β-carotene (0.228 µg/mL) (Fig. 1 (a, b and c, respectively)). This enhanced productivity highlights AZR1's greater photosynthetic efficiency and biosynthetic capacity, likely due to more effective utilization of organic substrates for ATP generation and carbon assimilation [10, 11]. Carotenoid synthesis peaked earlier than biomass, consistent with secondary metabolite production under nutrient-limiting conditions [12].

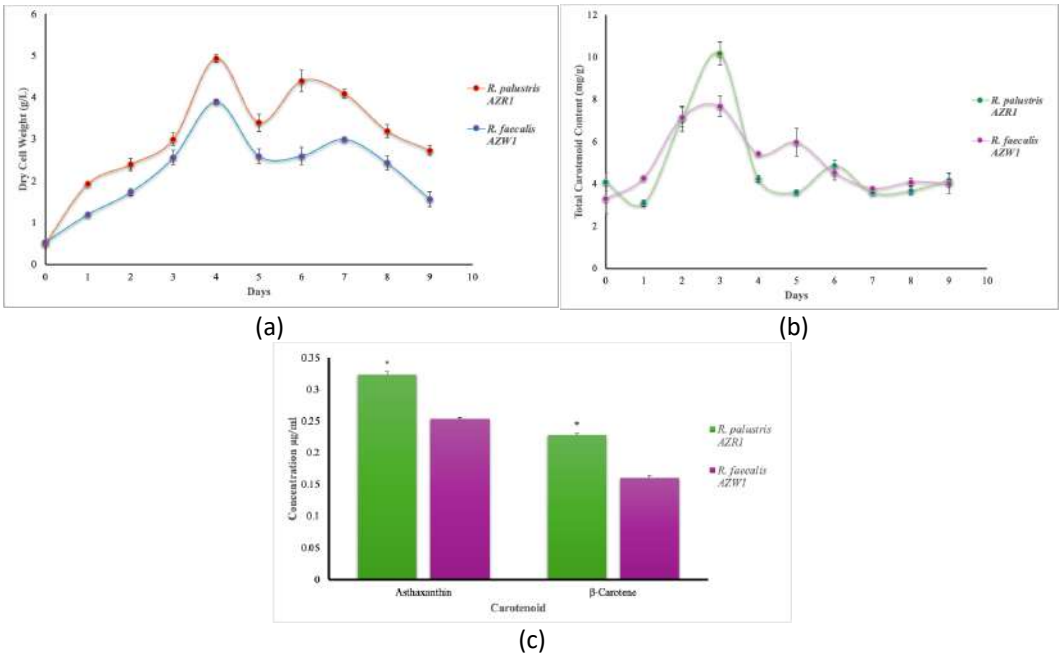

Fig. 1. (a) Dry cell weight (g/L); (b) total carotenoid production (mg/g) and (c) Concentration of astaxanthin and β-carotene in AZR1 and AZW1. Values are means ± SD; bars with * denote significant differences ($p < 0.05$)

Both strains exhibited a favorable nutritional profile with high crude protein (AZR1: 53.17%; AZW1: 50.55%) and low crude fibre (Fig. 2 (a)). AZR1 also had significantly higher total lipid content (7.77%) and distinct FAME profiles (Fig. 2 (b-d)). AZR1 showed consistently higher concentrations of Saturated Fatty Acids (SFA) and Monounsaturated Fatty Acids (MUFA), dominating in C18:1ω7 (790.26 µg/mL). While overall Polyunsaturated Fatty Acids (PUFA) were higher in AZR1, the profile of AZW1 differed significantly ($p < 0.05$) this results consistent with prior reports on *R. palustris* [13]. Their fatty acid profiles were dominated by Monounsaturated Fatty Acids (MUFAs), but critically, both produced detectable levels of essential PUFAs like ARA, underscoring their value as a nutritionally complete supplement compared to conventional feeds [14].

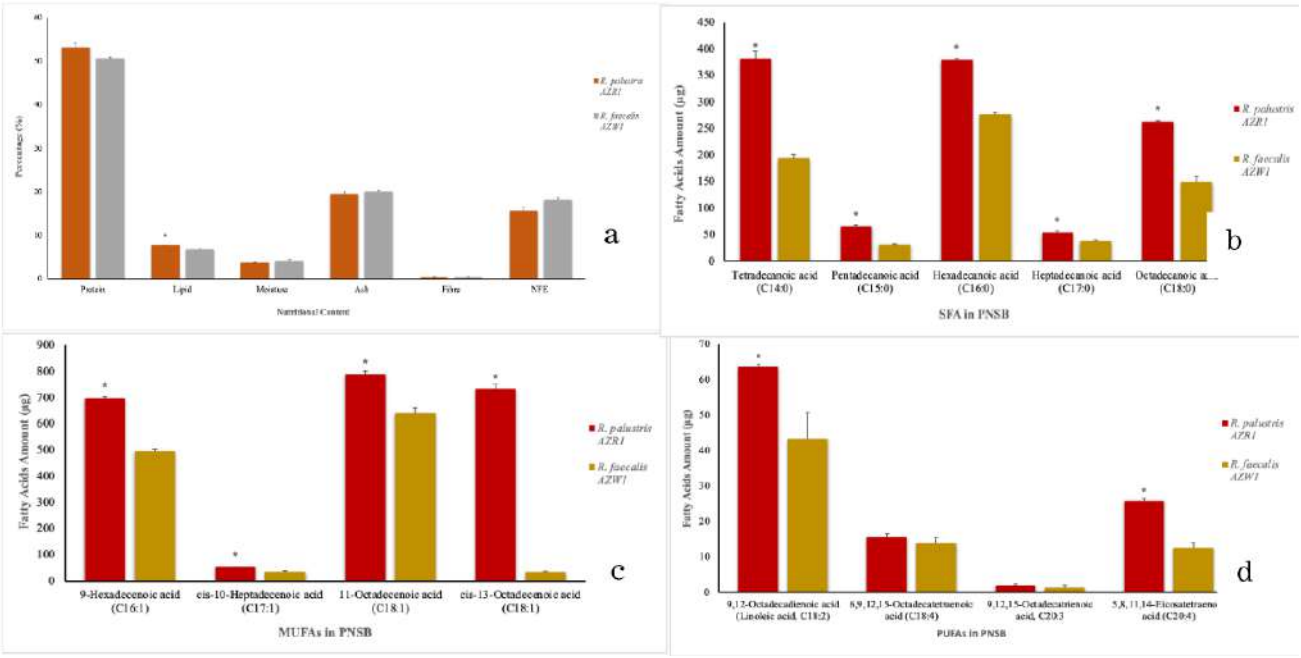

Fig. 2 Nutritional Contents of AZR1 and AZW1. (a) Proximate nutritional composition; (b) Saturated fatty acid (SFA); (c) Monounsaturated fatty acid (MUFA); (d) Polyunsaturated fatty acids (PUFAs) in AZR1 and AZW1 (% dry weight) with * denote significant differences ($p < 0.05$)

3.2 Artemia Performance and Enrichment

3.2.1 Growth and Water Quality:

Dietary supplementation with AZR1 significantly enhanced Artemia growth in both gnotobiotic (final length: 9.60 mm) and hatchery-scale (final length: 10.11mm) conditions, outperforming the yeast control and PKC Nutri+ treatments (Fig. 3 (a and b, respectively)). This is primarily attributed to the high digestibility of the PNSB cell wall [15, 16] and the transfer of essential nutrients and digestive enzymes [17]. Furthermore, AZR1 provided a significant biosecurity benefit, maintaining the lowest ammonium (NH_4^+) (≤ 2 mg/L) in the rearing tanks [18] compared to yeast and PKC Nutri+ (up to 8.0 mg/L), (Fig. 3 (c)) due to its ammonia assimilation and probiotic activity [19, 20]. The ease of PNSB digestion makes it a superior alternative to baker's yeast, which exhibits poor digestibility [21].

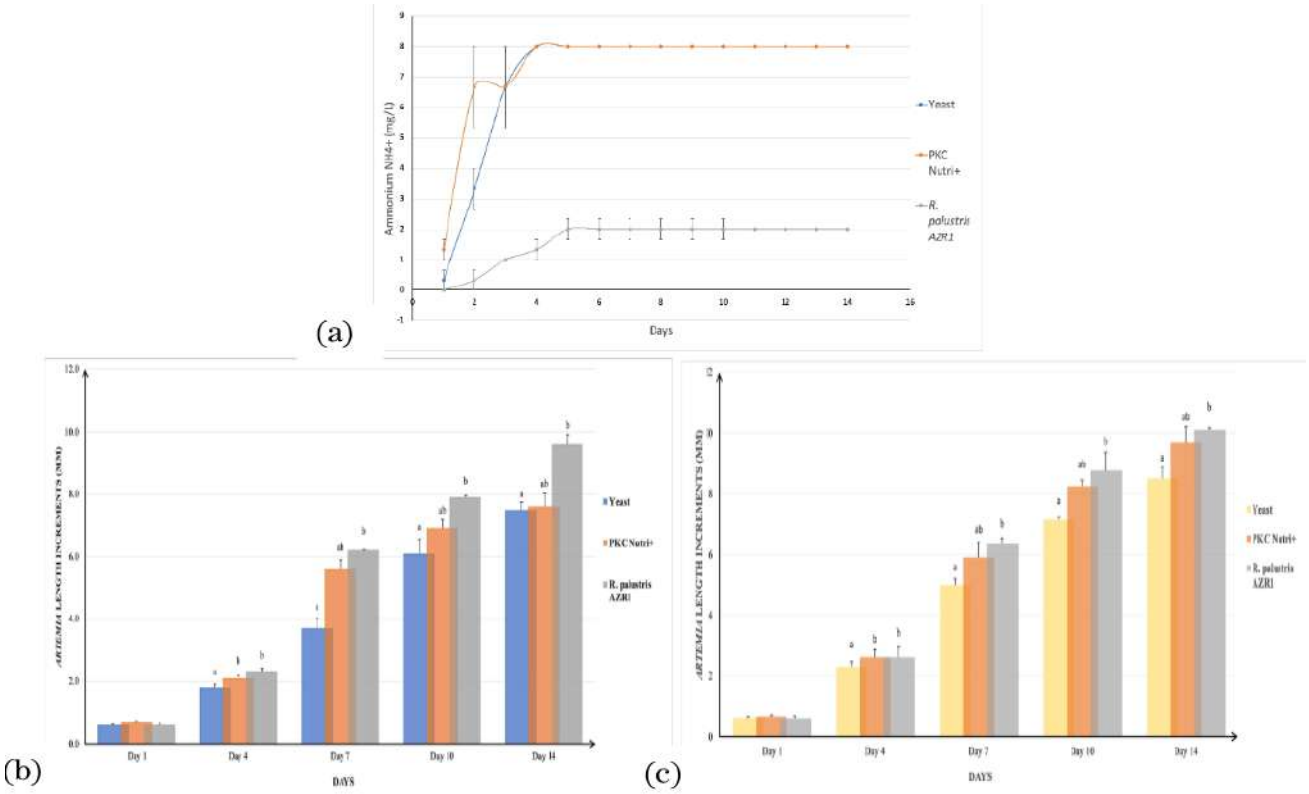

Fig. 3 Length increment (mm) of *Artemia* fed different diets under (a) gnotobiotic and (b) hatchery culture conditions over 14 days. While (c), Ammonium (NH_4^+) concentration (mg/L) in hatchery culture. Values are means ± SD (n = 3); bars with different letters indicate significant differences at each time point ($p < 0.05$)

3.2.2 Disease Resistance and Immunity

Following a lethal challenge with the pathogen *V. campbellii* (LC50: 1 x 108 CFU/mL), *Artemia* fed the AZR1 strain demonstrated significantly improved survival (91.67%) compared to both the yeast control (56.67%) and the PKC Nutri+ group (51.67%) (Fig. 4(a)). This marked resistance is directly attributed to the immunostimulatory effects of the AZR1 strain. Specifically, AZR1 induced a strong and sustained upregulation of key innate immune markers in *Artemia*. By Day 14, the expression of prophenoloxidase (proPO)-central to invertebrate defense-was upregulated by up to 4.72-fold [22, 23], and the stress protein Hsp70 was upregulated by up to 6.73-fold [24] (Fig. 4(b-c)). This "priming effect" indicates that the AZR1 probiotic acts as a potent immunostimulant, thereby enhancing *Artemia*'s inherent resilience against both biotic stressors, like *V. campbellii,* and abiotic stressors [25].

3.2.3 Nutritional and Pigment Enhancement

Feeding *Artemia* with the AZR1 strain significantly enhanced its macronutrient profile, resulting in the highest measured crude protein (50.96%) and total lipid (6.62%) content (Fig. 5(a)). Fatty Acid Methyl Ester (FAME) analysis further confirmed that the AZR1 diet significantly enriched the *Artemia* with Saturated Fatty Acids (SFA) and Monounsaturated Fatty Acids (MUFA) (Fig. 6(a-c)). While PKC Nutri+ showed superiority for PUFA enrichment (uniquely providing EPA) (Fig. 6(a)), the AZR1 treatment was highly effective in boosting overall nutritional quality (Fig. 6(a-c)).

Crucially, AZR1 proved to be a highly effective carotenoid delivery system. *Artemia* fed this strain exhibited pronounced reddish pigmentation, confirming efficient pigment transfer. HPLC quantification demonstrated the greatest accumulation of essential pigments, with the highest levels of astaxanthin (0.1942 µg/mL) and β-carotene (0.1189 µg/mL) recorded in the AZR1-fed group (Fig. 5(a-b)). As *Artemia* lacks the necessary enzymes to synthesize astaxanthin *de novo*, dietary supplementation is required [26, 27]. These results affirm that strain AZR1 functions not only as a nutritious SCP source but also as a superior carotenoid delivery mechanism, significantly enhancing the quality and value of *Artemia* as a live feed in aquaculture.

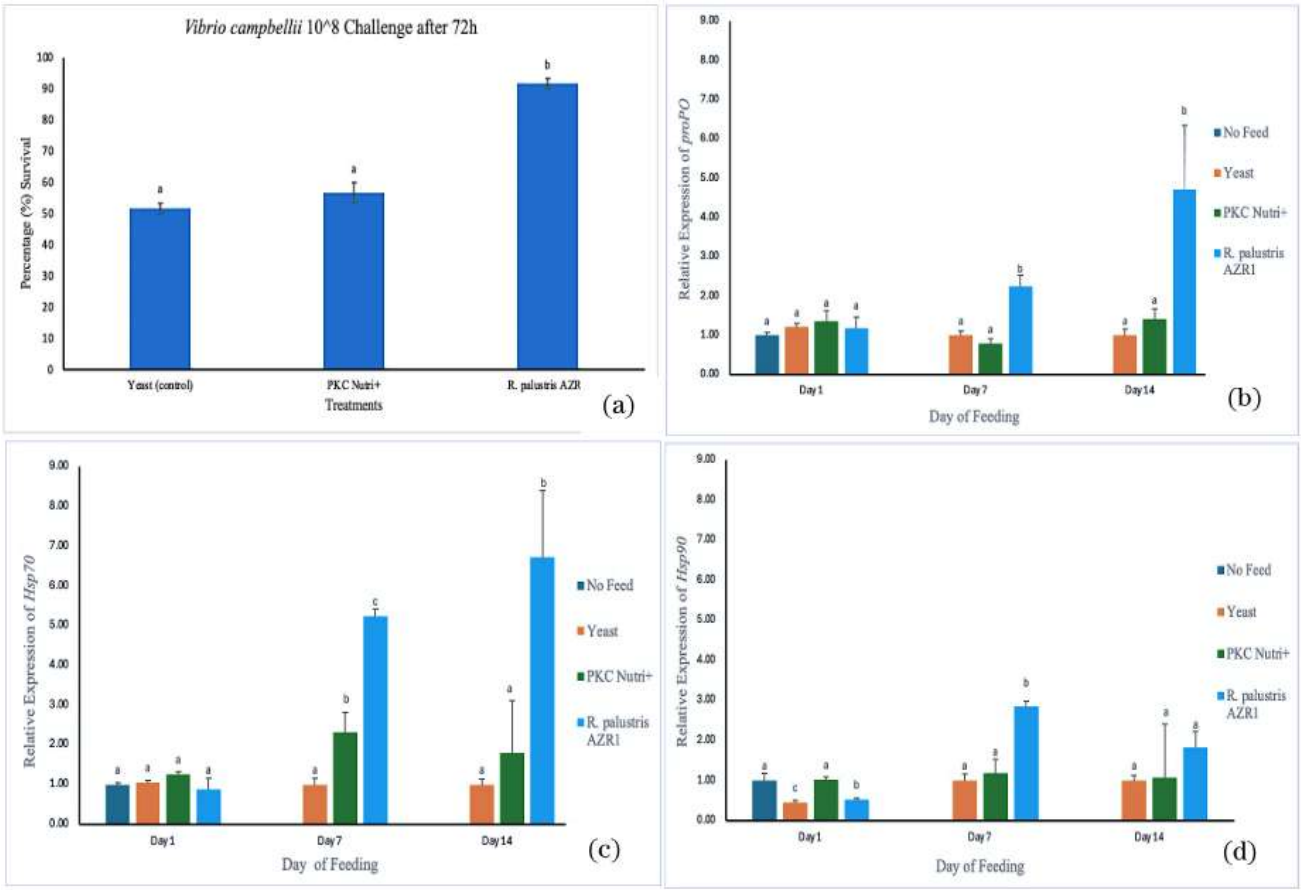

Fig. 4 (a) Survival percentage of Artemia fed different diets following 72-h exposure to V. campbellii at LC_{50} (1×10^8 CFU/mL). (b-d) Relative expression of (a) proPO, (b) Hsp70, and (c) Hsp90 in Artemia fed yeast, PKC Nutri+, and R. palustris AZR1 over 14 days. Values are means ± SD (n = 3); different letters denote significant differences ($p < 0.05$)

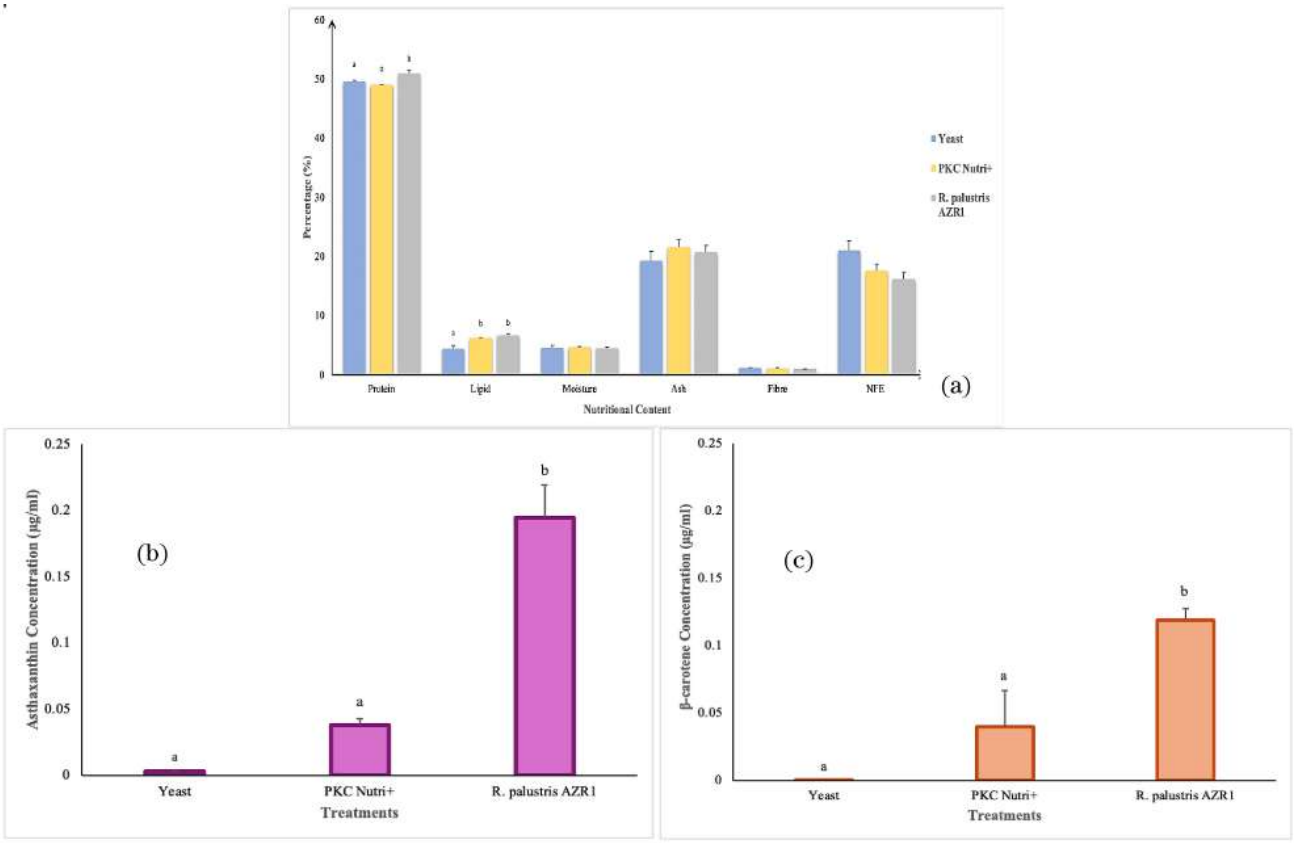

Fig. 5 (a) Proximate composition (% dry weight) of Artemia; (b-c) Concentrations of (a) astaxanthin and (b) β-carotene (µg/mL) in Artemia fed yeast, PKC Nutri+, and AZR1 for 14 days. Values are means ± SD (n = 3); bars with different letters indicate significant differences ($p < 0.05$)

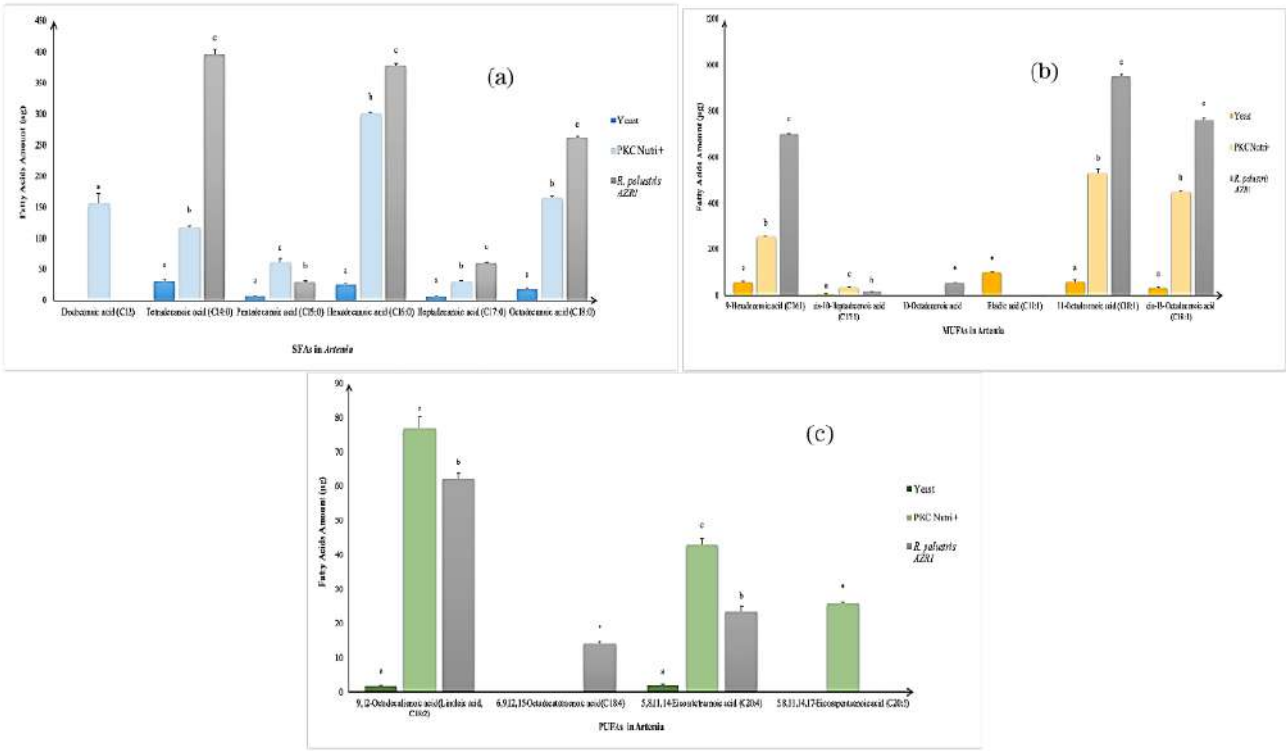

Fig. 6 (a) Saturated fatty acid (SFA); (b) Monounsaturated fatty acid (MUFA); (c) Polyunsaturated fatty acid (PUFA) profile (µg/mg dry weight) of Artemia fed different diets. Values are means ± SD; bars with different letters are significantly different ($p < 0.05$)

4. Conclusions

This study establishes *Rhodopseudomonas palustris* AZR1, a mangrove-derived PNSB, as a superior, multifunctional feed supplement for Artemia. We found that AZR1 not only achieved superior biomass and carotenoid production (specifically astaxanthin and β-carotene) but also translated these benefits directly to the Artemia host. When used as a live feed, AZR1 enhanced *Artemia* growth across gnotobiotic and hatchery systems, substantially improving its nutritional value (protein and lipid content) and pigmentation via efficient carotenoid transfer. Crucially, AZR1 demonstrated robust probiotic and immunomodulatory properties. Supplementation significantly upregulated key immune genes (proPO, Hsp70) and conferred enhanced survival against a *Vibrio campbellii* challenge. Furthermore, AZR1 improved culture water quality by substantially reducing ammonium accumulation. In summary, *Rhodopseudomonas palustris* AZR1 is a safe, sustainable, and effective Single-Cell Protein (SCP) source with demonstrated capacity for growth promotion, immunostimulation, and bioremediation. Its application is highly valuable for next-generation live feed strategies. Future work should focus on optimizing its LC-PUFA profile using cost-effective waste streams to maximize its potential within the circular bioeconomy.

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