Journal MVZ Cordoba
2022; september-december. 27(3):e2578.
https://doi.org/10.21897/rmvz.2578

Original

Biological, nutritional, and hematoimmune response in juvenile *Cherax quadricarinatus* (Decapoda: Parastacidae) fed with probiotic mixture

Yuniel Méndez-Martínez[1]* ; Yenny G. Torres-Navarrete[1] ; Edilmar Cortés-Jacinto[2] ; Marcelo U. García-Guerrero[3] ; Luis H. Hernández-Hernández[4] ; Danis M. Verdecia[5] .

[1]Universidad Técnica Estatal de Quevedo (UTEQ), Facultad de Ciencias Pecuarias y Biológicas, Quevedo, Los Ríos, Ecuador.
[2]Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Programa de Acuicultura, La Paz, BCS, Mexico.
[3]Instituto Politécnico Nacional, Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional–Santa Cruz Xoxocotlán, Oaxaca, Mexico.
[4]Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala, Tlalnepantla, Edo. de México, Mexico.
[5]Universidad de Granma, Facultad de Ciencias Agropecuarias, Bayamo, Granma, Cuba.
*Correspondence: ymendezm@uteq.edu.ec

Received: November 2021; Accepted: July 2022; Published: September 2022.

ABSTRACT

Objective. To evaluate the effect on biological, nutritional, and hematoimmune indicators of juvenile *Cherax quadricarinatus* were cultivated and fed with a probiotic mixture. **Materials and methods.** A completely randomized design (DCA) with six treatments: 0 (control), 1×10^2 µL, 2×10^2 µL, 3×10^2 µL, 4×10^2 µL and 5×10^2 µL of a probiotic mixture (Bacterol Shrimp Forte), with three repetitions each, 18 experimental tanks of diameter 1.7 m and area of 2.26 m^2 were used, with a density of 20 juveniles (0.95 ± 0.10g and 7.78 ± 0.77mm) per tank for 60 days. Biological (weight, length, weight gain, weight increase, specific growth rate, length gain, length increase and survival), nutritional (feed conversion, feed efficiency and protein efficiency rate) and hematoimmune (total of hemocytes, differential hemocytes, phagocytic rate, superoxide dismutase and hypoxic stress) parameters were measured. **Results.** For biological indicators, the best results ($p<0.05$) were obtained when using 4×10^2 µL of the probiotic (final weight: 9.11 g; final length: 68.95 mm; specific growth rate: 3.74). Regarding the nutritional parameters, the best results were found with for 3×10^2 µL (feed conversion: 1.09, feed efficiency: 0.91, and protein efficiency: 2.61); although there were no differences between 3×10^2 and 4×10^2 µL. For the hematoimmune response, there were differences ($p<0.05$) for all the indicators under study, with a better performance for 4×10^2 µL of the probiotic mixture. **Conclusions.** The probiotic mixture induces the hematoimmune, biological, and nutritional response with the best response for concentrations of 3×10^2 µL, 4×10^2 µL.

Keywords: Growth rate; hemocytes cells; hypoxic stress; phagocytic activity; survival (*Source: DeCS*).

How to cite (Vancouver).
Méndez-Martínez Y, Torres-Navarrete YG, Cortés-Jacinto E, García-Guerrero MU, Hernández-Hernández LH, Verdecia DM. Biological, nutritional, and hematoimmune response in juvenile *Cherax quadricarinatus* (Decapoda: Parastacidae) fed with probiotic mixture. Rev MVZ Cordoba. 2022; 27(3):e2578. https://doi.org/10.21897/rmvz.2578

ISSNL: 0122-0268

RESUMEN

Objetivo. Evaluar indicadores biológicos, nutricionales y hematoinmunes en juveniles *Cherax quadricarinatus* que fueron cultivados y alimentados con una mezcla de probióticos. **Materiales y métodos.** Un diseño completamente aleatorizado (DCA) con seis tratamientos: 0 (control), 1×10^2 µL, 2×10^2 µL, 3×10^2 µL, 4×10^2 µL y 5×10^2 µL de mezcla de probióticos (Bacterol Shrimp Forte), con tres repeticiones cada una; se utilizaron 18 tanques experimentales de diametro 1.7 m y área de 2.26 m^2, con una densidad de 20 juveniles (0.95±0.10 g y 7.78±0.77mm) por tanque durante 60 días. Se midieron los parámetros biológicos (peso, longitud, aumento de peso, aumento de peso, tasa de crecimiento específico, aumento de longitud, aumento de longitud y supervivencia), nutricionales (conversión alimenticia, eficiencia alimenticia y eficiencia proteica) y hematoinmune (total de hemocitos, hemocitos diferenciales, tasa fagocítica, superóxido dismutasa y estrés hipóxico). **Resultados.** Para los indicadores biológicos, los mejores resultados ($p< 0.05$) se obtuvieron al utilizar 4×10^2 µL del probiótico (peso final: 9.11 g; longitud final: 68.95 mm; tasa de crecimiento específico: 3.74). En cuanto a los parámetros nutricionales, los mejores resultados se obtuvieron con 3×10^2 µL (conversion alimenticia: 1.09, eficiencia alimenticia: 0.91 y eficiencia proteica: 2.61); aunque no hubo diferencias entre 3×10^2 y 4×10^2 µL. Para la respuesta hematoinmune, hubo diferencias ($p<0.05$) para todos los indicadores en estudio, con un mejor desempeño para 4×10^2 µL de la mezcla de probióticos. **Conclusiones.** La mezcla de probióticos induce la respuesta hematoinmune, biológica y nutricional con la mejor respuesta para concentraciones de 3×10^2 µL y 4×10^2 µL.

Palabras clave: Células hemocitos; estrés hipóxico; tasa de crecimiento; tasa fagocítica; supervivencia (Fuente: DeCS).

INTRODUCCIÓN

Aquaculture has become one of the fastest growing food production industry in Latin America (20%) and the world (7%) per year, significantly contributing to global food security and this growth has been extending continuously for more than two decades (1,2). After fish, crustaceans are placed as the second world aquaculture product.

Redclaw crayfish *Cherax quadricarinatus* is one of the cultivated species. It is native of northern Australia and to the southeast in Papua New Guinea (3), and it was introduced in various Latin America countries in the 90s. It is a specie adaptable to a wide range of weather conditions, with notable advantages due to its omnivorous eating habits, high growth rate, easy handling, adequate organoleptic characteristics, good meat quality rich in amino acids and fatty acids (4). It is appreciated in the market both for ornamental purposes and for human consumption (5).

However, in aquaculture, it is required to control stress and diseases and regardless of the species, the purpose of this is to ensure their health and productive yield (6). The use of prophylactic and therapeutic treatments (antibiotics) has been used to maximize the health and performance of cultured organisms. However, antibiotics have proven to have some disadvantages, as they penetrate into body tissues reducing their quality for consumption and causing health problems (7,8).

Antibiotics are also widely restricted for their impact on the environment (9) and because eventually, many pathogenic microorganisms become resistant (7,10). In this sense, the use of environmentally friendly food additives, such as probiotics, have becoming a good option as safe dietary supplements in the aquafeed industry, and are among the many strategies and alternatives to reduce the excessive use of chemotherapeutic agents such as antibiotics, developing the capacity for tolerance under stress conditions and improving the resistance and immunity of the host (11,12). They can also take advantage from their invertebrate host by fighting pathogens through a competitive exclusion mechanism (7,13), as well as promoting growth, survival and healthier animals (11,14). Probiotics commonly used in aquaculture include Gram-positive, Gram-negative bacteria, bacteriophages, yeasts, and single-celled algae (10,13,15).

Several studies have attributed an improvement in animal growth to the nutritional benefits of probiotic bacteria since they produce digestive enzymes and vitamins and make available minerals and trace elements (9). However, all

the modes of action discussed above require that the particular probiotic that want to be utilized, can successfully colonize the region in where its effect can occur successfully (10,12).

In recent years, *Bacillus* spp. (16) and yeasts (17) have been tested frequently in crustacean aquaculture. In the case of *Bacillus* spp., this microorganism can sporulate, grow rapidly and tolerate a wide range of conditions in host. They also have been proven very useful to improve water quality, reduce harmful bacteria amount in the culture and maximize the host response capacity without the addition of antibiotics (18). Furthermore, oral administration of yeast species, in particular *Saccharomyces cerevisiae*, has been shown to enhance the immune response in crustaceans (19).

Previously, many research involving the application of beneficial bacteria as probiotics have been carried out (13,14,15). From these researchs is evident that a mixture of several probiotics might produce better results (20). In this sense, the adding of mixed probiotics to the food has provided benefits in species such as *Macrobrachium rosembergii* (6). However, the dose-effect relationship of a probiotic must be carefully determined to avoid an overdose, which can cause a decrease in its groeing promoting effects and an increase in costs, or conversely, the use of very low doses could drastically reduce its efficiency on the cultured animals (14,20).

Despite the scientific advances, current knowledge on the use of probiotics as feed additives and the immune response in aquaculture is scarce for the initial stages of development in many species, like *Cherax quadricarinatus*. Considering the above, the purpose of this research was to evaluate several biological, nutritional, and hematoimmune indicators of juvenile redclaw *Cherax quadricarinatus* fed with a probiotic mixture.

MATERIALS AND METHODS

Study site. This research was executed in the Aquaculture Laboratory, of the Universidad Técnica Estatal de Quevedo (UTEQ), Quevedo, Los Ríos, Ecuador (01°03'18''S, 79°25'24''W), with an altitude of 120 meters above sea level with an average temperature of 24 °C.

Ethical considerations. The study was carried out strictly following the Standard Operating Procedures (SOP) for the Use of Experimental Animals of the UTEQ.

Treatments and feeding: Six treatments were used, which consisted of increasing doses of probiotic mixture: 0 (control), 1×10^2 µL, 2×10^2 µL, 3×10^2 µL, 4×10^2 µL and 5×10^2 µL / 100 L of water, respectively. The probiotic mixture (Bacterol Shrimp Forte, Dukay SA, CL) utilized contained strains of *Saccharomyces cerevisiae* (5×10^8 CFU / mL), *Lactobacillus acidophilus* (5×10^8 CFU / mL) and *Bacillus* spp. (5×10^8 CFU / mL). The mixture was incubated with molasses and water in a ratio of $1.5:10:3\times10^3$, respectively, for 24 hours, before being supplied directly into the water in the culture tanks every three days.

Juveniles were fed daily 5% of its live weight with basal pelleted diet, distributed in two portions (50% at 9:00 am and 50% at 5:00 pm). Basal pelleted diet was formulated (Table 1) and prepared according with Méndez-Martínez et al. (4,21) and the proximal analysis (dry matter, proteins, ethereal extract, nitrogen-free extract, ash, and crude energy) was performed according to methods of AOAC (22).

Experimental design: Juveniles *C. quadricarinatus* (0.95 ± 0.10 g and 7.78 ± 0.77 mm) were obtained under laboratory (UTEQ) conditions, acclimatized for a week before starting the experiment with a duration of 60 days.

A completely randomized design was applied of six treatments with 3 replications (experimental tanks) with 20 juveniles of *C. quadricarinatus* per tank, for a total of 360 juveniles and eighteen circular plastic tanks (diametric: 70 cm and area: 9.39 cm^2) filled with freshwater (200 L), respectively. The water in the experimental tanks were replaced (40%) every three days, to eliminate feces and food remains, and water quality parameters were monitored; to determine water temperature, a mercury thermometer was used, dissolved oxygen (DO) was determined with a digital oximeter (55-DO, YSI Incorporated, Yellow Springs, OH, USA). The pH, ammonia (NH_3 / NH_4), nitrites (NO_2-) and nitrates (NO_3-) were measured with colorimetric kits (Saltwater Master Test, OH, USA), respectively (21).

Table 1. Formulation and chemical composition of the pelleted diet.

Ingredients	%
Fish meal[1]	48.50
Soybean paste[2]	11.93
Sorghum flour[2]	10.65
Wheat flour[3]	13.90
Corn flour[2]	4.50
Soy Lecithin[3]	1.00
Fish oil[4]	1.52
Gelatin[3]	2.00
Calcium carbonate[3]	1.00
Choline chloride[3]	0.50
Mineral premixes[3] *	2.0
Vitamin premixes[3]--	2.0
Vitamin C[3]	0.50
Proximal composition real (% Dry Matter)	
Dry material	92.56
Ash	7.76
Ethereal Extract	7.40
Protein	35.20
Fiber	3.43
E. L. N. N †	38.77
Gross energy (MJ / g)	15.38

[1] Comercial El Gordillo, Santo Domingo de los Tsáchilas, Ecuador. [2] Valencia- Avícola, Valencia, Ecuador.[3]Supermaxi, Quevedo, Ecuador, [4] Productos Pesqueros S.A, Manta, Ecuador. *(mg/kg diet) Mineral premix: KCl. 0.5; $MgSO_4.7H_2O$. 0.5; $ZnSO_4.7H_2O$. 0.09; $MnCl_2.4H_2O$. 0.0234; $CuSO_4.5H_2O$. 0.005; KI. 0.005; $CoCl_2.2H_2O$. 0.0025; Na_2HPO_4. 2.37. --(mg/kg dieta) Vitamin premix: Vitamin B12 (0.02); Vitamin A acetate. 5000 IU; Vitamin D3. 4000 IU; α-tocopherol acetate 100 IU; menadione (5); thiamine HCl (60); riboflavin (25); pyridoxine HCl (50); folic acid (10); dl-capantothenic acid (75); nicotinic acid (5); biotin (1); inositol (5). † Nitrogen-free extract = 100 - (% crude protein +% ethereal extract +% crude fiber +% ash).

Temperature was maintained between 27.5 and 30 °C with the help of built-in thermostats (JAD Aquarrium Co., Guangdong, CN), respectively (21). The pH was kept between 7.0 and 8.0 and dissolved oxygen (DO) between 4.7 and 6.2 mg / L, ammonium from 0 to 0.25 ppm, nitrite at 0.01 ppm and nitrate with values from 0 to 10 ppm.

Biological and nutritional parameters. To determine the weight in juveniles, a digital scale was used (PE 3600 Mettler-Toledo, ± 0.01 g, Columbus, OH, USA), the length was evaluated with a vernier caliper (GT-MA15 Gester, ± 0.001 mm, Xiamen, CN). Animals were previously anesthetized for biometrics. The following variables were calculated (23):

Increased weight (%) = (final weight - initial weight) × 100
Weight gain (g) = (final weight - initial weight)
Specific growth rate = (logarithm of final weight - logarithm of initial weight / number of days) × 100
Longitude gain = (final length - initial length)
Length increase = (final length- initial length) × 100
Survival = (number of final organisms / number of initial organisms) × 100
Feed efficiency = (weight gain / feed consumption) × 100

Protein efficiency = (weight gain / protein consumption)

Feed conversion = (feed intake / weight gain)

Hematoimmune responses. At the end of the feeding trial, *C. quadricarinatus* juveniles were fasted during 14 hours prior to the extraction of hemolymph. For this, they were placed in dorsoventrally, exposing the ventral hemolymphatic sinus. The surface was disinfected with alcohol (90%). The hemolymph extraction was carried out with sterile syringes (27G × 13 mm) with a hypodermic needle between the first pair of pleopods. SIC-EDTA was used as anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM Hepes, 10 mM EDTA, pH 7.3) at 4 °C, to preserve a 2:1 ratio (2 volumes of SIC-EDTA per each extracted volume of hemolymph). The needle was inserted upwards, the hemolymph was extracted and immediately homogenized to prevent coagulation (24).

Then, the samples were placed in sterile 1.5 mL Eppendorf polypropylene microtubes and labeled. Subsequently, they were diluted in a ratio of 3:1 (150 µL of 4% formaldehyde per 50 µL of the anticoagulant and hemolymph mixture), which were stored at 4 °C to perform the total count of hemocytes and differentials (hyaline, semi-grainy and grainy) (25). For the analysis of phagocytic activity, superoxide dismutase (SOD) and oxyhemocyanin, hemolymph without formaldehyde was used.

Phagocytic activity was measured in agreement with Hauton (26) and Chen et al. (27). Fresh hemolymph (40 µL) was spread on glass slides. Then, slides were incubated until the hemolymph was dried out. Meanwhile, a zymosan working solution was prepared by dissolving 0.0125 g of powdered zymosan in 25 mL of sterile

seawater. Zymosan solution (40 µL) was added to the dried hemolymph samples on the slide and air dried. Then, they were treated with a 10% formaldehyde solution (seawater solvent) for 20 min. Subsequently, the glass slides were transferred to a GIEMSA solution and incubated during 20 min for cell staining of hemocytes.

To determine the oxyhemocyanin concentration, 20 µL of hemolymph diluted with 80 µL of SIC-EDTA was added and the absorbance at 335 nm was read with a spectrophotometer (28,29). The SOD enzyme activity was determined using 10 µL of hemolymph and a commercial kit (Ransel, Randox, Crumlin, Antrim, UK) based on the principle of the oxidation of glutathione (GSH) by cumene hydroperoxide catalyzed by GPx with glutathione reductase (GR) and NADPH. The absorbance was read at 340 nm.

At the end of the bioassay, hypoxia stress was determined. For this, 18 plastic containers of 200 mL each and six organisms were used for each test. Each container was considered a repetition. The elimination of dissolved oxygen (<0.1 mg / L) in the water was assured by adding sodium bisulfite ($NaHSO_3$) (0.15 g / 500 ml). Juveniles were exposed to hypoxia for 1 h. Samplings were carried out every 5 minutes and the number of living and dead organisms were recorded.

Statistical analysis. Bartlett and Kolmogorov-Smirnov tests were applied. A simple Analysis of Variance (ANOVA) ($p \leq 0.05$) was then applied to the different levels of probiotic mixture, which was the only source of variation. For the analysis of differences between means, a Tukey's test was applied. All statistical processing ($p \leq 0.05$) were performed with the statistical program 14.0v (InfoStat®, Cordova, AR).

RESULTS

Biological indexes in present research for tested juveniles are presented in Table 2. Final weight, weight increase, weight gain, specific growth rate, final length, length increase, and length gain in treatments using 3×10^2 µL and 4×10^2 µL of the probiotic mixture there was no statistical difference, but there was a significant difference ($p < 0.05$) compared to the control and the others treatments, respectively. Survival was significantly higher ($p < 0.05$) (88.33%) for the treatment with 3×10^2 µL of the probiotic mixture.

Table 2. Biological and nutritional indicators in juveniles of *Cherax quadricarinatus* fed with a mixture of probiotics for 60 days.

Indicators	Probiotic mixture (x 10^2 µL)						SE±	P-value
	0	1	2	3	4	5		
IW (g)	0.97	0.93	0.93	0.96	0.96	0.94	0.055	0.567
IL (mm)	7.95	7.56	7.58	7.8	7.84	7.68	0.995	0.494
FW (g)	6.21^{d}	7.33^{c}	7.51^{c}	8.85^{a}	9.11^{a}	8.28^{b}	1.967	0.001
FL (mm)	47.26^{e}	58.71^{c}	55.37^{d}	67.62^{a}	68.95^{a}	63.52^{b}	3.567	0.001
SGR	3.09^{d}	3.44^{c}	3.48^{c}	3.71^{a}	3.74^{a}	3.62^{b}	0.040	0.001
WI (%)	523.98^{d}	640.25^{c}	658.17^{c}	789.23^{a}	815.01^{a}	733.10^{b}	10.34	0.001
WG (g)	5.24^{d}	6.40^{c}	6.58^{c}	7.89^{a}	8.15^{a}	7.33^{b}	0.578	0.001
LI (%)	65.53^{e}	85.24^{c}	79.65^{d}	99.71^{a}	101.84^{a}	93.07^{b}	3.789	0.001
LG (mm)	39.32^{e}	51.14^{c}	47.79^{d}	59.83^{a}	61.10^{a}	55.84^{b}	2.456	0.001
FC	1.54^{d}	1.33^{c}	1.27^{bc}	1.09^{a}	1.13^{ab}	1.24^{bc}	0.150	0.011
FE	0.65^{d}	0.75^{c}	0.79^{c}	0.91^{a}	0.89^{ab}	0.81^{bc}	0.110	0.012
PE	1.87^{d}	2.15^{c}	2.24^{c}	2.61^{a}	2.53^{ab}	2.31^{bc}	0.280	0.010
SV (%)	66.67^{e}	71.67^{d}	83.33^{b}	88.33^{a}	81.67^{b}	76.67^{c}	4.673	0.020

SE ± = Standard Error. ^{abcde} Different superscripts differ significantly (p <0.05), within the rows. IW: Initial Weight, IL: Initial Length, FW: Final Weight, FL: Final Length, SGR: Specific Growth Rate, WI: Weight Increase, WG: Weight Gain, LI: Length Increase, LG: Length Gain, FC: Feed Conversion, FE: Feed Efficiency, PE: Protein Efficiency, SV: Survival.

Nutritional parameters (feed conversion, feed efficiency and protein efficiency) are presented in Table 2. No significant differences were found ($p<0.05$) between the treatments with 3×10^2 µL and 4×10^2 µL of the probiotic mixture, but there were differences between the control and all others.

Significant differences on hematoimmune response are presented in Table 3. Total hemocytes (9.27 and 11.74 million cells / mL) and hyaline hemocyte cells (28.30 and 25.86%) showed a better response ($p<0.05$) with in the treatments with 3×10^2 and 4×10^2 µL of the probiotic mixture, respectively. A higher granulocyte hemocyte cell count (33.17%) and phagocytosis rate (38.28%) was observed ($p<0.05$) in the 4×10^2 µL treatment, respectively. While semi-granulocytes were higher in the control with 60.79%. For oxyhemocyanin, the highest ($p<0.05$) values were 0.84 and 0.82 mmol / L, when 2×10^2 µL and 3×10^2 µL of a probiotic mixture were applied, higher ($p<0.05$) in comparison with the control.

Table 3. Hematoimmune indicators in juveniles of *Cherax quadricarinatus* fed with a mixture of probiotics for 60 days.

Indicators	Probiotic mixture (x 10^2 µL)						SE±	P-value
	0	1	2	3	4	5		
TH (million cells /mL)	4.39^{d}	6.35^{c}	5.84^{c}	9.27^{ab}	11.74^{a}	8.16^{b}	2.17	0.034
HHC (%)	15.71^{c}	15.50^{c}	21.72^{b}	28.30^{a}	25.86^{a}	21.41^{b}	3.64	0.031
SHC (%)	60.79^{a}	54.22^{b}	51.39^{b}	42.79^{c}	41.30^{c}	52.66^{b}	4.22	0.012
GHC (%)	23.50^{d}	30.27^{b}	26.88^{bc}	28.91^{b}	33.17^{a}	25.93^{c}	2.46	0.016
PR (%)	15.89^{d}	18.64^{d}	29.61^{b}	24.87^{c}	38.28^{a}	26.44^{bc}	3.37	0.043
OX (mmol/L)	0.66^{c}	0.63^{c}	0.84^{a}	0.82^{a}	0.73^{b}	0.71^{b}	0.21	0.006

SE ± = Standard Error. ^{abcd} Different superscripts differ significantly (p <0.05), within rows. HT: Total Hemocytes, CHH: Hyaline Hemocyte Cells, CHS: Semi Granulocyte Hemocyte Cells, CHG: Granulocyte Hemocyte Cells, TF: Phagocytosis Rate, OX: Oxyhemocyanin.

When analyzing hypoxic stress survival percentages of SOD activity (Figures 1 and 2), significant differences ($p<0.05$) were found depending on dosage. The highest hypoxia survival values were found in the 4×10^2 µL treatment with 76.19%. It should be noted that increases of 19.05, 57.15, 47.62, 66.67 and 57.15% (1, 2, 3 and 4×10^2 µL of probiotic mixture) were obtained in comparison with the control. While the enzyme SOD maintained a similar trend. Its highest value were with the concentration of 2×10^2 µL (41.18 Unit / mL), although with this concentration did not had significant differences in comparison with 4×10^2 µL of the probiotic mixture.

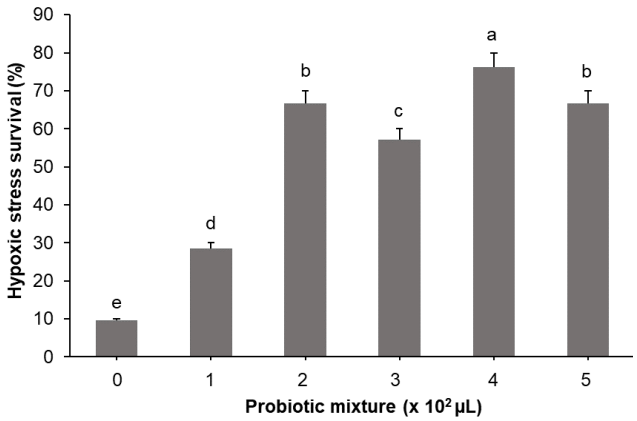

Figure 1. Hypoxia stress survival (± SE) in juveniles of *Cherax quadricarinatus* fed with a mixture of probiotics for 60 days.

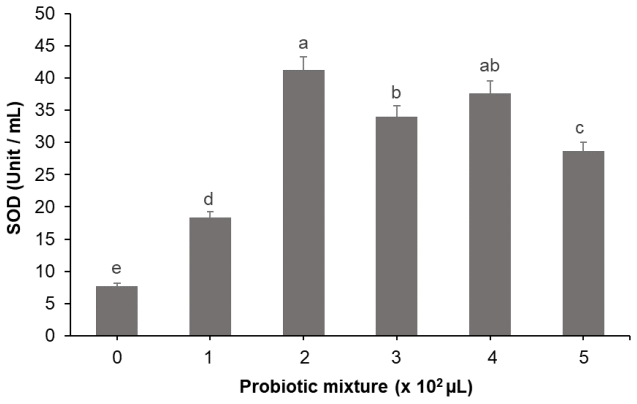

Figure 2. SOD enzyme activity (± SE) in juveniles of *Cherax quadricarinatus* fed with a mixture of probiotics for 60 days.

DISCUSSION

In aquaculture, as well as in other growing industries, the use of probiotic organanisms is having a wide acceptance since it is useful to improve resistance, to treat infections and diseases as well as the improvement of growth, survival, and water quality (6,12).

During aquaculture practices, the accumulation of organic matter affects water quality and cause pollution. It also allow pathogens that grow inside the culture. Water quality in our study were in agreement with the standards for freshwater decapod crustaceans including *C. quadricarinatus* (23,30,31,32).

Biological and nutritional parameters in present research were influenced by the dose of the probiotic mixtures as a result of the synergistic effect between the strains and its secondary metabolites. This effect can be beneficial since probiotic strains not only synthesize extracellular enzymes such as proteases, amylases, and lipases, but also other compounds required for growth (vitamins, amino acids, and fatty acids), which contribute to the absorption of nutrients more efficiently (15). This has been previously proven by Seenivasan et al (6), when supplementing with different doses of probiotic, reported survival (90%), growth (1.04 g), growth rate (0.88%), feed conversion (1.57 g), and protein efficiency (1.38%) significantly higher regarding the control treatment. Some other studies have shown positive effects of probiotics, such is the case of *Bacillus* sp. and *Clostridium* sp. on the growth of *M. rosenbergii*, where these food additives have been demostrate to improve digestion, assimilation, and metabolism of nutrients in crustaceans and fish by promoting the synthesis of digestive enzymes, improving growth and survival (33).

Some previous research had tested the use of probiotics with *C. quadricarinatus,* where Amrullah and Wahidah (14) found increases in body weight up to 7.30 g, with 2.63 g higher than the control treatment and with a survival of 73% while feeding the species with diets supplemented with three different concentrations of the probiotic mixture of *Micrococcus* spp. This effect can be caused by the symbiotic behavior of probiotics, besides stimulating the microbiota of the digestive tract with a more efficient intestinal biota and modifying the selection of bacterial enzymes (11).

Pérez-Chabela et al (33) found that using a mixture with strains of *Bacillus subtilis*, *B. licheniformis* and *B. subtilis*, supplied in the food as probiotics to *Litopenaeus vannamei* juveniles, improves growth rates by increasing the concentration of the probiotic mixture. Madani et al (34), when evaluating the effect of mixing probiotics (*Bacillus subtilis* and *Bacillus licheniformis*) in *L. vannamei* larvae, found that the addition of the probiotic mixture to the food had a positive effect on growth, which was confirmed by Zhao et al (35) in *Macrobrachium rosenbergii*, finding beneficial effects on biological and nutritional parameters, as well as an improvement on the action of digestive enzymes and optimization of costs.

Table 2 and 3 shows that probiotics improved growth and nutrient uptake because of their ability to stimulate beneficial intestinal microbiota, also with hematoimmuno-regulatory effects (10). In *C. quadricarinatus*, Amrullah and Wahidah (14), found that mixtures of probiotics with *Micrococcus* spp., increased total hemocytes, hyaline hemocytes, semi-granulocytes and granulocytes, as well as phagocytosis activity. This is a reason of an increasing in immunological activity caused by the activation of the non-specific immune response. Hemocytes are a reliable indicator to determine and prevent diseases, as well as a marker of the physiological status of the animal, since hemocytes in crustaceans is the basis of the immune system as they perform phagocytosis, encapsulation and lysis of unwanted cells (16).

Hemocytes play a vital role in defense, hyaline cells are responsible for phagocytosis, semi-granules also play a role in phagocytosis, encapsulation and in the release of the prophenoloxidase system. Furthermore, they synthesize and release peneidins and peptides, the granulated cells store the enzymes that constitute the prophenoloxidase system at a higher level than the semi-granular ones, just as they synthesize and store the peneidins, they intervene in encapsulation; which have been demonstrated to be an important part of the innate immune system (17). Zhao et al (35), when using different concentrations of *Bacillus pumilus* in *M. rosenbergii*, they did not find differences in the total hemocyte count, while for phagocytic activity (37%), differences were highly significant.

Valipour et al (36), used *Lactobacillus plantarum* in *Astacus leptodactylus* to evaluate its effect on the immune response, finding increases of 1.2×10^6 cells / mL and 1.4×10^6 cells / mL for total hemocyte and hyaline hemocyte cell count, respectively. Azad et al (37) evaluated the effect of probiotics on the immunological competence of *M. rosenbergii* against *Vibrio* spp. and *Aeromonas* spp., finding that prawns treated with probiotics improved the hematoimmune parameters and total hemocyte count (11×10^5 cells / mL), hyaline hemocyte cells (79%) and semigranulocyte hemocyte cells (19%). Such results differ from those obtained in this work, which can be attributed to species, experimental conditions, type of probiotic or experimental design. Results obtained indicate a greater capacity to prevent the invasion of foreign particles. It is known that hemocytes are also involved in different physiological functions, including carbohydrate metabolism, transport and storage of proteins and amino acids, stress regulation, leading to a disease resistance (13,15).

In present study, SOD activity was significantly influenced ($p<0.05$) by the probiotic concentration. Since nutritional status is the most important factor influencing immune defense mechanisms, low or high quantities of nutrients can alter the immune system, causing cell stress (32). In this sense, Soberanes-Yepiz et al (38), found that high antioxidant activity are the consequence of multiple oxidative reactions and therefore, an index of a high production of free radicals. In present research, the use of probiotics can be an important tool as an immunostimulant to prevent diseases, given the role played by beneficial bacteria in the prevention of the spreading of pathogens.

Ranjit-Kumar et al (39), when using different concentrations of *Bacillus licheniformis* as a probiotic, found an increased superoxide dismutase (SOD) and antibacterial activity (76%), which confirms such assumptions. In decapoda crustaceans, the level of SOD decreases during pathogenic infection, since its effect on the delay of the normal activity of cells of the hepatopancreas and hemocytes. Hence, the increase in SOD activity with probiotic supplementation is an index of positively regulated immune status.

In conclusion, probiotic mixtures induce an hematoimmune response, improving the biological and nutritional indicators. Such effect is best at at concentrations of 3×10^2 µL and 4×10^2 µL.

Conflict of interests

All authors declare that during the preparation and preparation of this work there was no conflict of interest.

Acknowledgments

Our thanks to L. Ramos and D. Zapatier, for the technical support. The research was supported by the Universidad Técnica Estatal de Quevedo. The Support Research Project is from the call FOCYCYT- 6th, project: PFOC6-22-20.

REFERENCES

1. Sonnenholzner-Varas JI. ¿Hacia dónde va la acuicultura de equinodermos en América Latina? Potencial, retos y oportunidades. Rev Biol Trop. 2021; 69(S1):514-549. https://doi.org/10.15517/rbt. v69iSuppl.1.46393

2. FAO. FishStatJ tool for fishery statistics analysis, Release 2.0.0. Universal software for fishery statistical time series. Global capture and aquaculture production: Quantities 1950-2019; Aquaculture values 1984-2019. Food and Agriculture Organization (FAO) Fisheries Department, Fishery Information, Data and Statistics Unit. Rome; 2021. https://www.fao.org/fishery/statistics/software/fishstatj/en

3. Numes AL, Zengeya TS, Hoffman AC, Measey GJ, Wey OLF. Distribution and establishment of the alien Australian redclaw crayfish, *Cherax quadricarinatus*, in South Africa and Swaziland. PeerJ. 2017; 5:e3135. https://doi.org/10.7717/peerj.3135

4. Méndez-Martínez Y, Ceseña CE, Luna-González A, García-Guerrero MU, Martinez-Porchas M, Campa-Cordova AI, et al. Effects of different dietary protein-energy ratios on growth, carcass amino acid and fatty acid profile of male and female *Cherax quadricarinatus* (von Martens, 1868) pre-adults. Aquac Nutr. 2021. 00: 1-16. https://doi.org/10.1111/anu.13379

5. Norshida I, Mohd-Nasir MSA, Khaleel AG, Sallehuddin AS, Syed Idrus SN, Istiqomah I, et al. First wild record of Australian redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) in the East Coast of Peninsular Malaysia. Bioinvasions Rec. 2021; 10(2):360–368. https://doi.org/10.3391/bir.2021.10.2.14

6. Seenivasan C, Saravana Bhavan P, Radhakrishnan S. Effect of probiotics (Binifit™) on survival, growth, biochemical constituents and energy budget of the freshwater prawn *Macrobrachium rosenbergii* post larvae. Elixir Aquaculture. 2011; 41:5919-5927. https://www.elixirpublishers.com/index.php?route=product/search&filter_name=macrobrachium&filter_type=Anywhere

7. Sapcharoen P, Rengpipat S. Effects of the probiotic *Bacillus subtilis* (BP 11 and BS 11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei*. Aquac Nutr. 2013; 19(6):946-954. https://doi.org/10.1111/anu.12040

8. Peredo AM, Buentello A, Gatlin DMIII, Hume M. Evaluation of a Dairy-Yeast Prebiotic in the Diet of Juvenile Nile Tilapia, *Oreochromis niloticus*. J World Aquac Soc. 2015; 46:92–101. https://doi.org/10.4025/actascianimsci.v42i1.47960

9. Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A, et al. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environ Microbiol. 2013; 15(7):1917-1942. https://doi.org/10.1111/1462-2920.12134

10. Kuebutornye FK, Abarike ED, Lu Y. A review on the application of Bacillus as probiotics in aquaculture. Fish Shellfish Immunol. 2019; 87:820-828. https://doi.org/10.1016/j.fsi.2019.02.010

11. Sumon MS, Ahmmed F, Khushi SS, Ahmmed MK, Rouf MA, Hasan-Chisty MA, et al. Growth performance, digestive enzyme activity and immune response of *Macrobrachium rosenbergii* fed with probiotic *Clostridium butyricum* incorporated diets. J King Saud Univ Sci. 2018; 30(1):21-28. http://dx.doi.org/10.1016/j.jksus.2016.11.003

12. Foysal J, Fotedar R, Siddik MA, Chaklader R, Tay A. *Lactobacillus plantarum* in black soldier fly (*Hermetica illucens*) meal modulates gut health and immunity of freshwater crayfish (*Cherax cainii*). Fish Shellfish Immunol. 2021; 108: 42-52. https://doi.org/10.1016/j.fsi.2020.11.020

13. Wee WC, Mok CH, Romano N, Ebrahimi M, Natrah I. Dietary supplementation use of Bacillus cereus as quorum sensing degrader and their effects on growth performance and response of Malaysian giant river prawn *Macrobrachium rosenbergii* juvenile towards *Aeromonas hydrophila*. Aquac Nutr. 2018; 24(6):1804-1812. https://doi.org/10.1111/anu.12819

14 Amrullah A, Wahidah W. Immune response and growth performance of crayfish *Cherax quadricarinatus* fed with synbiotic supplemented diet. JAI. 2019; 18(1):33-45. https://doi.org/10.19027/jai.18.1.33-45

15. Rebecca M, Gao Q, Sun C, Liu B, Song C, Adisu D, et al. Effect of dietary *Clostridium butyricum* and different feeding patterns on growth performance, antioxidant and immune capacity in freshwater prawn (*Macrobrachium rosenbergii*). Aquac Res. 2020; 52(1):12-22. https://doi.org/10.1111/are.14865

16. Ambas I, Fotedar R, Buller N. Health Status of Marron, *Cherax cainii* (Austin, 2002) Fed Customized Probiotic *Bacillus mycoides*. J Aquac Mar Biol. 2017; 6(4):00165. http://dx.doi.org/10.15406/jamb.2017.06.00165

17. Phupet B, Pitakpornpreecha T, Baowubon N, Runsaeng P, Utarabhand P. Lipopolysaccharide- and β-1, 3-glucan-binding protein from *Litopenaeus vannamei*: purification, cloning and contribution in shrimp defense immunity via phenoloxidase activation, Dev Comp Immunol. 2018; 81:167-179. https://doi:10.1016/j.dci.2017.11.016

18. Kuebutornye FKA, Abarike ED, Lu Y. A review on the application of Bacillus as probiotics in aquaculture. Fish Shellfish Immunol. 2019; 87:820-828. https://doi.org/10.1016/j.fsi.2019.02.010

19. Babu DT, Antony SP, Joseph SP, Bright AR, Philip R. Marine yeast *Candida aquaetextoris* S527 as a potential immunostimulant in black tiger shrimp *Penaeus monodon*. J Inverte Pathol. 2013; 112(3):243–252. https://doi.org/10.1016/j.jip.2012.12.002

20. Vine NG, Leukes WD, Kaiser H. Probiotics in marine larviculture. FEMS Microbiol Rev. 2016; 30(3):404-427. https://doi.org/10.1111/j.1574-6976.2006.00017.x

21. Méndez-Martínez Y, Pacheco-Morales GK, Del Barco-Ibarra KA, Torres-Navarrete YG, Hernández-Vergara MP. Respuesta bioquímica e inmune en tilapia roja (*Oreochromis mossambicus* × *O. niloticus*) con suplementación de quitosano en dieta. Rev Fac Agron Luz. 2021; *38*(4), 1016-1034. https://doi.org/10.47280/RevFacAgron(LUZ).v38.n4.15

22. AOAC (Association of Official Agricultural Chemists). Official methods of analysis of AOAC International. 21st ed., Rockville, MD, USA: AOAC; 2019. https://www.aoac.org/wp-content/uploads/2019/08/Front-Matter-List-of-Changes-2.pdf

23. Méndez-Martínez Y, García-Guerrero MU, Arcos-Ortega FG, Martínez-Córdova LR, Yamasaki-Granados S, Pérez-Rodríguez JC, et al. Effect of different ratios of dietary protein-energy on growth, body proximal composition, digestive enzyme activity, and hepatopancreas histology in *Macrobrachium Americanum* (Bate, 1868) prawn juveniles. Aquaculture. 2018; 485:1–11. https://doi.org/10.1016/j.aquaculture.2017.11.012

24. Vargas-Albores F, Guzman MA, Ochoa JL. A lipopolysaccharide binding agglutinin isolated from brown shrimp (*Penaeus californiensis* Holmes) haemolymph. Comp Biochem Physiol. 1993; 104:407-413. https://doi.org/10.1016/0305-0491(93)90387-K

25. Johansson M, Keyser P, Sritunyalucksana K, Söderhäll K. Crustacean haemocytes and haematopoiesis. Aquaculture. 2000; 191:45-52. https://doi.org/10.1016/S0044-8486(00)00418-X

26. Hauton C L. The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). Comp Biochem Physiol B, Biochem Mol Biol. 1998; 119(4):619–623. https://doi.org/10.1016/S0305-0491(98)00036-4

27. Chen H, Mai K, Zhang W, Liufu Z, Xu W, Tan B. Effects of dietary pyridoxine on immune responses in abalone, *Haliotis discus hannai* Ino. Fish Shellfish Immunol. 2005; 19(3):241–252. https://doi.org/10.1016/j.fsi.2004.12.006

28. Hagerman L. Haemocyanin concentration of juvenile lobsters (*Homarus gammarus*) in relation to moulting cycle and feeding conditions. Mar Biol. 1983; 17:11-17. https://doi.org/10.1007/BF00393205

29. Chen W, Cheng JC. Effects of pH, temperature and salinity on immune parameters of the freshwater prawn *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 2000; 10: 387-391. https://doi.org/10.1006/fsim.2000.0264Ç

30. Jones CM, Valverde C. Development of Mass Production Hatchery Technology for the Redclaw Crayfish, *Cherax quadricarinatus.* Freshw Crayfish. 2020; 25(1):1–6. https://doi.org/10.5869/fc.2020.v25-1.001.

31. Gainza O, Romero J. Manano oligosacáridos como prebióticos en acuicultura de crustáceos. Lat Am J Aquat Res. 2017; 45(2):246-260 https://doi.org/10.3856/vol455-issue2-fulltext-2

32. Paul P, Rahman A, Ghosh A. Observation of probiotics effect on the growth, survival and production of giant freshwater prawn (*Macrobrachium rosenbergii*) in south-west part of Bangladesh. Int J Biosci. 2019; 14(3):45-53. http://dx.doi.org/10.12692/ijb/14.3.45-53

33. Pérez-Chabela ML, Alvarez-Cisneros YM, Soriano-Santos J, Pérez-Hernández MA. Los probióticos y sus metabolitos en la acuicultura. Una Revisión. Hidrobiológica. 2020; 30(1):93-105. https://doi.org/10.24275/uam/izt/dcbs/hidro/2020v30n1

34. Madani NSH, Adorian TJ, Ghafari-Farsani H, Hoseinifar SH. The effects of dietary probiotic Bacilli (*Bacillus subtilis* and *Bacillus licheniformis*) on growth performance, feed efficiency, body composition and immune parameters of whiteleg shrimp (*Litopenaeus vannamei*) postlarvae. Aquac Res. 2018; 49:1926-1933. https://doi.org/10.1111/are.13648

35. Zhao C, Zhu J, Hu J, Dong X, Sun L, Zhang X, et al. Effects of dietary Bacillus pumilus on growth performance, innate immunity and digestive enzymes of giant freshwater prawns (*Macrobrachium rosenbergii*). Aquac Nutr. 2019; 25(3):712-720. https://doi.org/10.1111/anu.12894

36. Valipour A, Nedaei S, Noori A, Asghar A, Hossein S. Dietary *Lactobacillus plantarum* affected on some immune parameters, air exposure stress response, intestinal microbiota, digestive enzyme activity and performance of narrow clawed crayfish (*Astacus leptodactylus*, Eschscholtz). Aquaculture. 2019; 504:121-130. https://doi.org/10.1016/j.aquaculture.2019.01.064

37. Azad MAK, Islam SS, Sithi IN, Ghosh AK, Banu GR, Bir J, et al. Effect of probiotics on immune competence of giant freshwater. prawn *Macrobrachium rosenbergii*. Aquac Res. 2018; 50(2):644-657. https://doi.org/10.1111/are.13942

38. Soberanes-Yepiz ML, Méndez-Martínez Y, García-Guerrero MU, Ascencio F, Violante-González J, García-Ibañez S, et al. Superoxide dismutase activity in tissues of juvenile cauque river prawn (*Macrobrachium americanum* Bate, 1868) fed with different levels of protein and lipid. Lat Am J Aquat Res. 2018; 46(3):543-550. https://doi.org/10.3856/vol46-issue3-fulltext-7

39. Ranjit-Kumar N, Prakash-Raman R, Jadhao SB, Kumar-Brahmchari R, Kumar K, Dash G. Effect of dietary supplementation of Bacillus licheniformis on gut microbiota, growth and immune response in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Aquacult Int. 2013; 21:387-403. https://doi.org/10.1007/s10499-012-9567-8