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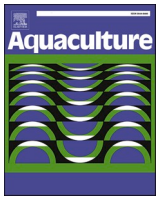

# Dietary supplementation of *Chlorella vulgaris* improved growth performance, immunity, intestinal microbiota and stress resistance of juvenile narrow clawed crayfish, *Pontastacus leptodactylus* Eschscholtz, 1823

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ABSTRACT

A 63- day feeding trial was conducted to study the effect of fishmeal substitution levels with *Chlorella vulgaris* meal (25, 50, 75 and 100%) on the growth performance, nutrient digestibility, immune responses and the stress resistance of juvenile narrow clawed crayfish, *Pontastacus leptodactylus* (6.19 ± 0.13 g). The highest values of final weight (25.52 g), specific growth rate (2.24% body weight day$^{-1}$), protein efficiency ratio (3.24), the protein productive value (54.67%), *in vivo* $ADC_{OM}$ (88.40%) and *in vivo* $ADC_{CP}$ (93.50%) and the lowest feed conversion ratio (1.49) were observed in the juvenile crayfish fed the diet containing 75% fishmeal substituted level with chlorella meal ($p < 0.05$). With an increment in the dietary inclusion level of chlorella meal, the activities of alkaline protease ($r^2 = 0.97$), lipase ($r^2 = 0.98$), amylase ($r^2 = 0.97$), phenoloxidase ($r^2 = 0.98$), superoxide dismutase ($r^2 = 0.99$), lysozyme ($r^2 = 0.96$) and nitric oxide synthase ($r^2 = 0.98$) were promoted as a third-order polynomial regression model ($p < 0.05$). There was an increasing trend in Lactobacillus count ($r^2 = 0.99$) and a decreasing trend in *Escherichia coli* count ($r^2 = 0.97$) with an increase in the fishmeal substitution level with chlorella meal. Based on the broken line regression model, the dietary fishmeal substitution level (%) with chlorella meal for maximum growth (SGR) and weight gain values and minimum FCR value of crayfish were estimated to be 85.49%, 78.14% and 78.13%, respectively.

## 1. Introduction

The share of finfish production decreased gradually from 97.2% to 91.5% during 2000–2018, while shellfish production (*e.g.* shrimps, crabs and crayfish) enhanced (FAO, 2020). World aquaculture production of freshwater crayfish was reported to be 1.71 million metric tonnes in 2018 (FishStat, 2020). The high valuable market size of crayfish with a worth of 9.6 billion $ in 2019 and the predicting an annual growth at a rate of 2.5% during the period 2020–2025 has attracted special attention for the economic production of crayfish in the modern aquaculture systems (IndustryARC, 2021). In this regard, a rising trend in the production of inland water aquaculture in Asia, excluding China, was reported from 32 to 48 million metric tonnes during 2010–2018 (FAO, 2020). This can be related to the use of unconventional brackish and saline waters, the improvement of rearing methods, new designs of cultivation systems, breeding of aquatic species, and mainly, special attention to the formulation and processing techniques of aquatic diets.

Recently, one of the challenges of sustainable production in the aquaculture industry, especially in crayfish farming (astaciculture), is to find replaceable feed ingredients with fishmeal due to the variable quality, processing methods and price fluctuations. Different protein sources are used in the aquatic diet production industry including animal-origin sources (marine or terrestrial ones), plant-origin sources (meal, protein concentrate and isolate) and single cell-origin sources (microalgae, fungi, yeasts and bacteria) (Ahmad et al., 2020; Araújo et al., 2021; Lunda et al., 2020; Safari, 2011; Safari et al., 2014b).

Nowadays, the inclusion of different forms (live, fresh paste and freeze/spray-dried powder) of marine microalgae (*e.g. C. vulgaris*,

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*Spirulina platensis, Haematococcus pluvialis* and Schizochytrium sp) as a sustainable and environmentally friendly protein source has received special attention in the aquatic diet production industry (Ahmad et al., 2020; Bélanger et al., 2021; Roy and Pal, 2015). *C. vulgaris*, a green unicellular alga, is widely used in the diet formulations of finfish and shellfish species as a protein-rich feed ingredient or a natural colorant (Ahmad et al., 2020; Araújo et al., 2021; Bito et al., 2020; Roy and Pal, 2015). Chlorella market size is expected to gain market growth of 7% in the forecast period of 2021 to 2025 with a worth of 284 million $ (More, 2021). Chlorella products is rich in crude protein (50–70%), crude fat (7–20%), carbohydrates (5–42%), dietary fibers (7–18%), minerals, vitamins and pigments (Ahmad et al., 2020; Bito et al., 2020). The chemical composition of chlorella meal in the terms of suitable profiles of amino acids and essential fatty acids, has made it as a feed ingredient that can be used instead of fishmeal in the aquatic diet production industry. However, chemical composition of chlorella products depends on medium, pH level, salinity, temperature and light intensity/duration (Ahmad et al., 2020). It has been confirmed that dietary inclusion of chlorella products in addition to providing the required nutrients has some biological effects such as antibacterial, bacterial quorum sensing, antihypertensive, antihypercholesterolemic, antihyperlipemic, antidiabetic, antioxidant, immunostimulant, stress resistance, intestinal microbiota booster, and gut health ameliator (Ahmad et al., 2020; Bito et al., 2020).

Partial fishmeal replacement with *C. vulgaris* was reported in the diet of giant freshwater prawn, *Macrobrachium rosenbergii* (Maliwat et al., 2017; Radhakrishnan et al., 2015; Radhakrishnan et al., 2014), Pacific white shrimp, *Litopenaeus vannamei* (Pakravan et al., 2018), Indian white shrimp, *Fenneropenaeus indicus* (Maliwat et al., 2017), crucian carp, *Carassius auratus* (Maliwat et al., 2017) and olive flounder, *Paralichthys olivaceus* (Rahimnejad et al., 2017). These positive effects can be related to the suitable profile of amino acids, long-chain polyunsaturated fatty acids (18:3n-3, 20:5n-3, 22:6n-3), pigments (*e.g.* β-Carotene and astaxanthin), vitamins (*e.g.* A, $B_1$, $B_2$, $B_6$, C and E), functional carbohydrates and bioactive peptides in chlorella meal (Ahmad et al., 2020; Bito et al., 2020). Although, it is very important to consider the species of chlorella, production methods (out door or indoor), processing techniques, type of aquatic species (*e.g.* finfish and shellfish), dietary regimes (*e.g.* herbivore, omnivore and carnivore) of tested species, initial weight, feeding period, and nutritional history. To our best knowledge, there is no information available on using chlorella meal in the diet of crayfish. In order to produce more economical diets of crayfish, we conducted studies on the determination of apparent digestibility coefficients of commonly used feed ingredients (Safari et al., 2014b) and evaluating the effect of using different feed additives including nucleotides (Safari et al., 2015a, 2015b), prebiotics (Safari et al., 2014a), synbiotics (Safari et al., 2017; Safari and Paolucci, 2017a), encapsulated organic salts (Safari et al., 2020), L-carnitine (Safari et al., 2015a, 2015b) and plant-based materials (Safari and Paolucci, 2017a) on growth performance, immune responses, hemolymph and biological challenge during the last decade. The aim of the present study was to assess the effect of fishmeal replacement with *C. vulgaris* meal on the growth performance, nutrient digestibility, immune responses and the stress resistance of juvenile narrow clawed crayfish (*Pontastacus leptodactylus*).

**Table 1**
The ingredient and chemical composition of the experimental diets of juvenile crayfish (6.19 ± 0.13 g) with 0% (control), 25%, 50%, 75% and 100% substitution of *C. vulgaris* meal with fishmeal.

| Ingredient (g $kg^{-1}$) | *C. vulgaris* | Experimental diets | | | | |
|---|---|---|---|---|---|---|
| | | 0 (Control) | 25 | 50 | 75 | 100 |
| Fishmeal[1] | | 40.5 | 30.25 | 20.01 | 9.76 | 0 |
| Chlorella meal[2] | | 0 | 10.25 | 20.49 | 30.74 | 40.99 |
| Soybean meal[1] | | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 |
| Corn gluten[1] | | 12 | 12 | 12 | 12 | 12 |
| Wheat flour[1] | | 10 | 10 | 10 | 10 | 10 |
| Corn starch[1] | | 10 | 10 | 10 | 10 | 10 |
| Fish oil[1] | | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 |
| Canola oil[1] | | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 |
| Soy lecithin[1] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Glucosamine[3] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Cholesterol[4] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Choline chloride[4] (70%)[4] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin C (stay)[4] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin premix[4, *] | | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Mineral premix[4, *] | | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Antifungus[1] | | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Filler (Carboxymethyl cellulose[3]) | | 4.5 | 4.5 | 4.5 | 4.5 | 4.11 |
| Ytterbium oxide[3] | | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Chemical composition (g $kg^{-1}$) | | | | | | |
| Dry matter | 918 | 845.27 | 845.25 | 845.31 | 845.34 | 845.32 |
| Crude protein | 584 | 380.5 | 380.1 | 380.3 | 380.3 | 380.6 |
| Crude fat | 39 | 133.9 | 133.2 | 132.5 | 131.8 | 131.3 |
| Crude fiber | 24 | 26.6 | 27.2 | 27.9 | 28.5 | 29.2 |
| Nitrogen free extract | 221 | 265.17 | 276.35 | 271.91 | 267.74 | 265.99 |
| Ash | 73 | 39.1 | 28.4 | 32.7 | 37.0 | 38.43 |
| Gross energy (Mj $kg^{-1}$) | 17.32 | 14.59 | 14.57 | 14.56 | 14.54 | 14.49 |

*Mineral premix contains (mg $Kg^{-1}$) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant, 100; *Vitamin premix contains (mg $Kg^{-1}$) E, 30; K, 3; Thiamine, 2; Riboflavin, 7; Pyridoxine, 3; Pantothenic acid, 18; Niacin, 40; Folacin, 1.5; Choline, 600; Biotin, 0.7 and Cyanocobalamin, 0.02.

[1] Saramad Fish Aquafeed Co, Iran.

[2] Abzygostaran Toloe-Pazh Co., Iran.

[3] Sigma, Germany.

[4] Kimia Roshd Co., Iran.

## 2. Material and methods

### 2.1. Experimental diets

A basal diet (380 g $kg^{-1}$, crude protein; 133.9 g $kg^{-1}$, crude fat; 14.59 Mj $kg^{-1}$, Gross energy) as control diet (Safari et al., 2014c) was formulated with winfeed software (WinFeed Limited, Cambridge, UK) (Table 1). *C. vulgaris* meal was kindly prepared from Abzygostaran Toloe-Pazh Co. (Khorasan Razavi province, Iran). The proximate composition of *C. vulgaris* (dry matter; g $kg^{-1}$) contained 918 g $kg^{-1}$ dry matter, 584 g $kg^{-1}$ crude protein, 39 g $kg^{-1}$ crude fat, 24 g $kg^{-1}$ crude fiber, 221 g $kg^{-1}$ nitrogen free extract, 73 g $kg^{-1}$ ash and 17.32 Mj $kg^{-1}$. Five experimental diets were formulated with different levels of *C. vulgaris* substituted with fishmeal including 0 (control diet), 25%, 50%, 75% and 100% (Table 1). Therefore, the inclusion level of chlorella meal in the experimental diets was 0, 10.25, 20.49, 30.74 and 40.99%, respectively (Table 1). After grinding the feedstuffs until reaching suitable particle size (lower than 250 μ), the mash was extruded (Fardan Machine Shargh CO, Khorasan Razavi, Iran) with mesh size 2 mm. Then, fish oil was coated over the pellet, dried at 30 °C, packed in three-layer waterproof nylon bags and maintained at −20 °C until use (Hardy and Barrows, 2002).

### 2.2. Crayfish and sample collection

Three hundred thirty healthy juvenile crayfish (6.19 ± 0.13 g) were obtained from the Shahid Yaghoobi reservoir (35° 9′ 36″N 59° 24′ 18″E, Khorasan Razavi Province, Iran), acclimatized for 14 days with rearing conditions and stocked at a density of twenty-two crayfish per 1000-L tank (2 × 1 × 0.5 m) in a semi-recirculating system with daily water exchange rate of 30% at three replicates for each experimental diet. Each tank was fitted with 22 plastic tubes (4 cm diameter and 12 cm length), which served as hiding places for the animals. Unconsumed feed was collected manually *via* siphoning voided feces three hours after feeding and weighed. Water temperature was maintained at 25.5 °C throughout the feeding trial. DO (6.35 ± 0.16 mg $l^{-1}$), pH (7.11 ± 0.32), hardness (138 ± 6.1 mg $l^{-1}$ as $CaCO_3$), unionized ammonia (<0.06 mg $l^{-1}$) and nitrite contents (<0.6 mg $l^{-1}$) were evaluated every week. Animals were held under Light: Darkness 14:10 h. Briefly, each diet was randomly assigned to a tank of crayfish and they were fed 3.5% body weight three times daily (8:00, 14:00 and 20:00) for 63 days. Biometry was done during first and last day of the experiment.

### 2.3. Evaluation of growth performance and carcass quality

At the end of the feeding trial, each crayfish was individually weighed (±0.01 g) on an electronic scale (AND, Japan). All parameters were corrected based on the ingested feed. Growth parameters, survival rate and nutrient efficiency indices were calculated as follows (Glencross et al., 2007; Safari et al., 2014c):

$$\text{Specific Growth Rate}\left(\text{SGR};\%\text{day}^{-1}\right) = \left[\left(\ln W_f - \ln W_i\right)/t\right] \times 100$$

$$\text{Survival Rate}\,(\%) = (\text{Final Individual Numbers}/\text{Initial Individual Numbers}) \times 100$$

$$\text{Voluntary Feed Intake}\left(\text{VFI};\%\text{body weight day}^{-1}\right) = \left[\left(\text{Feed}_{\text{consumed (DM)}}\right)/\left(W_{\text{mean}} \times t\right)\right]$$

$$\text{Feed Conversion Ratio (FCR)} = \left(\text{Feed}_{\text{consumed}}/W_{\text{gain}}\right)$$

$$\text{Protein Efficiency Ratio (PER)} = \left(W_{\text{gain}}/\text{Crude protein}_{\text{consumed}}\right)$$

$$\text{Protein Productive Value}\,(\text{PPV};\%) = 100 \times \left[\left(\text{Protein}_{\text{retained}}\right)/\left(\text{Protein}_{\text{consumed}}\right)\right]$$

In the above equations, $W_i$, $W_f$, $W_{mean}$ and $W_{gain}$, t and $Feed_{consumed}$ are initial weight, final weight, mean weight, weight increment (g), time period (day) and consumed feed (g), respectively.

### 2.4. Calculation of in vivo apparent nutrient digestibility

*In vivo* apparent digestibility coefficients (ADCs) of organic matter ($ADC_{OM}$), crude protein ($ADC_{CP}$), crude fat ($ADC_{CF}$) and gross energy ($ADC_{GE}$) of experimental diets were calculated according to the following equations (Safari et al., 2014a):

$$\text{ADC}_{\text{test}} = 100 \times \left(1 - \left(\text{Marker}_{\text{test}} \times \text{Nutrient}_{\text{feces}}/\text{Marker}_{\text{feces}} \times \text{Nutrient}_{\text{test}}\right)\right)$$

In above equation, the terms $Marker_{test}$ and $Marker_{feces}$ represent the marker (0.1 g $kg^{-1}$ Ytterbium oxide, $Yb_2O_3$, in the diet) contents of the diet and feces, respectively, and $Nutrient_{test}$ and $Nutrient_{feces}$ represent the nutritional parameters of concern (*e.g.* protein or energy) in the diet and feces, respectively.

### 2.5. Biochemical analyses

#### 2.5.1. Hemolymph indices

In the 63th day, five crayfish from each tank (15 crayfish per treatment) were killed after 24 h of last feeding time. All assays were done one by one at three replicates. According to protocol previously described (Safari et al., 2014c); hemolymph was collected from ventral sinus with needle (25G), pooled and stored *via* two following methods: (1) 1 ml microtube without anticoagulant agent and (2) 1 ml microtube containing 0.4 ml Alsever as an anticoagulant. Briefly, 125 μl- plasma obtained from the second method was used to measure the following hemolymph indices: THC with hemocytometer cell (Beco, Hamburg, Germany) (Jiang et al., 2004), hyaline count (HC), semi- and large-granular count (SGC and LGC, respectively) *via* hemolymph extension at room temperature (25 °C), fixation at methanol for 1 min, staining with the method of May- Grunwald- Giemsa and then, counting with light microscope (Pousti and Adib Moradi, 2004). Total plasma protein was estimated using the biuret procedure.

#### 2.5.2. The activities of phenoloxidase, superoxide dismutase, lysozyme and nitric oxide synthase

The remaining anticoagulated hemolymph (250 μl) was centrifuged (700 ×*g* for 20 min at 4 °C) to separate the hemocytes from plasma, and the supernatant fluid was used for plasma determinations (Safari et al., 2014c; Zhang et al., 2011). All activities of enzymes were standardized based on the protein concentration. Phenoloxidase activity (PO) was assayed spectrophotometrically by recording the formation of dopachrome from *L*-dihydroxyphenylalanine (L-DOPA) at final reading 490 nm (Hernández-López et al., 1996; Safari et al., 2014c). Superoxide dismutase (SOD) activity was measured by observing the inhibition of ferricytochrome C reduction at final reading 550 nm (Cooper et al., 2002). The lysozyme (LYZ) activity was determined with a decrease in absorbance compared to *Micrococcus lysodeikticus* suspension without plasma at final reading 530 nm (Ellis, 1990). Nitric oxide synthase (NOS) activity was measured with the assay kit (Nanjing Jiancheng Bioengineering Institute, China) (Marzinzig et al., 1997).

#### 2.5.3. Digestive enzyme activities

On the 63th day, live crayfish (15 individuals per treatment) were transported to laboratory, anesthetized with ice, and dissected with scalpel according to method lastly explained *(Safari* et al., 2014c). The hepatopancreas was removed, rinsed with distilled water, dried with paper towel and, homogenized (30 g/70 ml distilled water) by a homogenizer (DI 18 Disperser). The homogenate was then centrifuged (at 10000 ×*g*, 4 °C, 25 min), and the supernatant was stored in liquid nitrogen. All hepatopancreas tissues were collected one by one without pooling. The measurement of digestive enzyme activities were explained elsewhere (Safari et al., 2014c). Briefly, the amylase activity was

measured using starch as substrate at final reading 550 nm with a UV/*VS* spectrophotometer (Ultro Spec 2000 Pharmacia Biotech) (Coccia et al., 2011). Lipase activity was measured using α-naphthyl caprylate as substrate at final reading 540 nm (López-López et al., 2003). Alkaline protease activity was determined using azocasein as substrate at final reading 366 nm (Fernández Gimenez et al., 2001). In this study specific enzyme activity was defined as enzyme units (U) per mg of protein.

### 2.6. Chemical analysis

Analysis of dry matter (oven drying, 105 °C), crude protein (N × 6.25, Kjeldahl system: Buchi Labortechnik AG, Flawil, Switzerland), crude fat (Soxtec System HT 1043: Foss Tecator, AB), ash (muffle furnace, 550 °C), gross energy (Parr bomb calorimetry model 1266, Parr Instrument Co., Moline, IL) and crude fiber (after digestion with $H_2SO_4$ and NaOH) analysis of feedstuffs, diets and feces were performed according to standard methods (AOAC, 2005). Nitrogen free extract (NFE) was calculated by subtraction dry matter minus crude protein, crude fat, crude fiber and ash contents. Organic matter was calculated by subtraction dry matter minus ash content. Ytterbium oxide was determined in diets and feces by inductively coupled plasma atomic absorption spectrophotometry (ICP; GBC Integra XL, Australia).

### 2.7. Bacteriological analysis

As described previously (Safari et al., 2014c), at the end of the experiment, crayfish (12 individuals per a treatment) was transported alive to laboratory, anesthetized with ice, rinsed with benzalkolium chloride (0.1% for 60 min) and dissected with scalpel. The microbial counts of total bacterial aerobic, lactobacillus, fungi and *E. coli* were determined in the digestive tract of the crayfish fed on experimental. Then, hepatopancreas was removed, homogenized with sodium chloride (0.9 *w/v*) using a homogenizer (DI 18 Disperser) and the homogenate was then centrifuged at 5000 ×*g*, 4 °C, for five min. Then, a 100 μL aliquot of each prepared sample was plated onto plate count agar (Merck, Germany), plate de Man, Rogosa, and Sharpe media (Merck, Germany), Potato Dextrose Agar (Merck, Germany) and Mac Conkey Agar (Merck, Germany) to determine total aerobic bacterial count, lactobacillus count, fungi count and *E. coli* count, respectively. Finally, the plates were incubated (25 °C for 5 days) and those containing 30–300 colonies were used for bacterial counting as colony forming units per gram (CFU $g^{-1}$) (Safari et al., 2017).

### 2.8. Bacterial exposure challenge

Challenge test was initiated on day 64 of the feeding trial. Twelve crayfish from each test diet-tank were injected with $1 \times 10^8$ cells $ml^{-1}$ *A. hydrophila*, ATCC 49141 through the base of the fifth thoracic leg with 2 ml bacteria stock suspension (Safari et al., 2014c; Sang et al., 2009). The injected crayfishes were marked before releasing back into their original tanks to avoid repeat-sampling. The infected crayfishes were not fed and monitored for survival rate after 48 h of injection.

### 2.9. Statistical analysis

All percentage data were transformed using arcsine method. After confirming the homogeneity of variance and normality of the data using Leaven and Kolmogorov-Smirnov tests (Zar, 2007), respectively, ANOVA were used to compare the treatments at three replicates. Duncan test was applied to compare significant differences among the treatments ($p < 0.05$) with SPSS™ version 19. Also, regression relations were done with XLSTAT 2012. Broken line regression model was done to determine the optimum fishmeal replacement level with chlorella meal. All results were given as mean ± SD.

## 3. Results

### 3.1. Growth performance and survival rate

Fishmeal substitution with different levels of chlorella meal (25, 50, 75 and 100%) improved significantly ($p < 0.05$) growth performance (final weight, FCR, VFI), survival rate and nutritional efficiency indices (PER and PPV) in juvenile crayfish compared with that of fed the control diet (Table 2; Fig. 1). Juvenile crayfish fed the 75%- chlorella meal diet showed the significantly ($p < 0.05$) highest values of final weight (25.52 g), SGR (2.25% BW $day^{-1}$), PER (3.24) and PPV (54.67%) and significantly ($p < 0.05$) lowest FCR value (1.49) (Table 2; Fig. 1).

Final weight ($r^2 = 0.77$), SGR ($r^2 = 0.98$), FCR ($r^2 = 0.99$), weight gain ($r^2 = 0.95$), PER ($r^2 = 0.98$) and PPV ($r^2 = 0.96$) increased significantly ($p < 0.05$) as third-order polynomial response to increased dietary chlorella meal level (Table 2, Figs. 1-2). Based on the broken line regression model, the dietary fishmeal substitution level (%) with chlorella meal for maximum growth (SGR) and weight gain values and minimum FCR value of crayfish were estimated to be 85.49%, 78.13% and 78.14%, respectively (Figs. 1-2).

### 3.2. In vivo apparent digestibility coefficients (ADCs) of organic matter, crude protein, crude fat and gross energy

With an increase in the dietary fishmeal substitution level with chlorella meal from 25 to 75%, the values of *in vivo* $ADC_{OM}$ increased from 66.37 to 88.40% (Table 2). The juvenile crayfish fed the 75% chlorella -diet showed the significantly ($p < 0.05$) highest values of *in vivo* $ADC_{CP}$ (93.50%) and *in vivo* $ADC_{GE}$ (91.51%) (Table 2). Feeding crayfish with the diets containing 75% and 100% fishmeal substitution levels with chlorella meal showed the significantly ($p < 0.05$) highest values of *in vivo* $ADC_{CF}$ (90.70–91.37%) (Table 2). The *in vivo* $ADC_{OM}$ ($r^2 = 0.95$), *in vivo* $ADC_{CP}$ ($r^2 = 0.91$), *in vivo* $ADC_{CF}$ ($r^2 = 0.99$) and *in vivo* $ADC_{GE}$ ($r^2 = 0.99$) increased significantly ($p < 0.05$) as a third-order polynomial response to increased fishmeal level substituted with dietary chlorella meal level in the crayfish diet (Table 2).

### 3.3. Hemolymph indices and challenge test

Feeding the juvenile crayfish with the diets containing different levels of fishmeal replaced with chlorella meal (25–100%), improved ($\times 10^5$ cell $ml^{-1}$) significantly ($p < 0.05$) the THC (123.33–183.33), HC (77–110), SGC (24–49) and LGC (29.67–54) than those of control diet (107, 68.67, 18.33 and 16.67, respectively) (Table 3). The THC ($r^2 = 0.98$), HC ($r^2 = 0.98$), SGC ($r^2 = 0.99$) and LGC ($r^2 = 0.99$) increased significantly ($p < 0.05$) as a third-order polynomial response to increased chlorella meal levels in the crayfish diet (Table 3). As shown in Fig. 3, the survival rate (%) of *A. hydrophila*- injected crayfish fed with the diets containing different levels of fishmeal replacement with chlorella meal (25–100%) were significantly ($p < 0.05$) higher than those of fed the control diet. Crayfish fed the 75% fishmeal substituted with chlorella meal showed the significantly ($p < 0.05$) highest survival rate after 48 h challenge test (Fig. 3).

### 3.4. The activities of phenoloxidase (PO), superoxide dismutase (SOD), lysozyme (LYZ) and nitric oxide synthase (NOS)

The juvenile crayfish fed the diets containing 25–100% fishmeal substituted levels with chlorella meal showed significantly ($p < 0.05$) higher activities of PO, SOD, LYZ and NOS than those of fed the control diet (Table 3). The activities of PO ($r^2 = 0.98$), SOD ($r^2 = 0.99$), LYZ ($r^2 = 0.96$) and NOS ($r^2 = 0.98$) increased significantly ($p < 0.05$) as a third-order polynomial response to fishmeal substitution with chlorella meal in the crayfish diet (Table 3).

**Table 2**
The mean (± SEM[1]) of initial weight (g), final weight (g), survival rate (%), specific growth rate (% body weight day$^{-1}$), feed conversion ratio, voluntary feed intake (% body weight day$^{-1}$), protein efficiency ratio, protein productive value (%), *in vivo* apparent digestibility coefficient (ADC) of organic matter ($ADC_{OM}$; %), crude protein ($ADC_{CP}$; %), crude fat ($ADC_{CF}$; %) and gross energy ($ADC_{GE}$; %) of crayfish fed the experimental diets with 0% (control), 25%, 50%, 75% and 100% substitution of *C. vulgaris* meal with fishmeal after 63 days ($n = 3$; Each replicate was stocked with 22 crayfish; $p < 0.05$)[2].

| | Control | Fishmeal replacement levels with *C. vulgaris* in diet (%) | | | | Pooled SEM | Regression analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 75 | 100 | | Regression formula | Adj. $r^2$ | *P*-value |
| Initial weight (g) | 6.19$^{a}$ | 6.19$^{a}$ | 6.19$^{a}$ | 6.18$^{a}$ | 6.20$^{a}$ | 0.006 | | | |
| Final weight (g) | 12.40$^{a}$ | 13.44$^{b}$ | 17.53$^{c}$ | 25.52$^{e}$ | 19.39$^{d}$ | 0.170 | 12.667–9.154*10$^{-5}$ × $^{3}$ + 0.013 × $^{2}$–0.268× | 0.94 | 0.0001 |
| Survival rate (%) | 70$^{a}$ | 90$^{b}$ | 93.33$^{bc}$ | 98.33$^{c}$ | 96.67$^{bc}$ | 2.236 | 70.381 + 5.330*10$^{-5}$ × $^{3}$–0.013 × $^{2}$ + 1.010× | 0.87 | 0.0001 |
| Specific growth rate (% body weight day$^{-1}$) | 1.10$^{a}$ | 1.23$^{b}$ | 1.66$^{c}$ | 2.25$^{e}$ | 1.81$^{d}$ | 0.007 | 1.1186–7*10$^{-6}$ × $^{3}$ + 0.001 × $^{2}$–0.0175× | 0.98 | 0.0001 |
| Feed conversion ratio | 3.15$^{e}$ | 2.69$^{d}$ | 1.99$^{c}$ | 1.48$^{a}$ | 1.78$^{b}$ | 0.015 | 3.1473 + 6*10$^{-6}$ × $^{3}$–0.0006 × $^{2}$–0.0053× | 0.99 | 0.0001 |
| Voluntary feed intake (% body weight day$^{-1}$) | 1.98$^{a}$ | 2.14$^{b}$ | 2.39$^{c}$ | 3.86$^{e}$ | 2.89$^{d}$ | 0.012 | 2.045–1.351*10$^{-5}$ × $^{3}$ + 0.002 × $^{2}$–0.046× | 0.82 | 0.0001 |
| Protein efficiency ratio | 1.36$^{a}$ | 1.78$^{b}$ | 2.33$^{c}$ | 3.24$^{e}$ | 3.08$^{d}$ | 0.015 | 1.387–6.453*10$^{-6}$ × $^{3}$ + 0.001 × $^{2}$–0.006× | 0.98 | 0.0001 |
| Protein productive value (%) | 28$^{a}$ | 36.33$^{b}$ | 44.67$^{c}$ | 54.67$^{e}$ | 51.00$^{d}$ | 1.000 | 28.243–7.289*10$^{-5}$ × $^{3}$ + 0.008 × $^{2}$ + 0.121× | 0.96 | 0.0001 |
| *In vivo* $ADC_{OM}$ (%) | 60.53$^{a}$ | 66.37$^{b}$ | 74.73$^{c}$ | 88.40$^{e}$ | 77.73$^{d}$ | 0.452 | 60.996 + 0.0001 × $^{3}$ + 0.018 × $^{2}$–0.228× | 0.95 | 0.0001 |
| *In vivo* $ADC_{CP}$ (%) | 76.80$^{a}$ | 79.70$^{b}$ | 81.40$^{c}$ | 93.50$^{e}$ | 92.50$^{d}$ | 0.115 | 77.301–6.347*10$^{-5}$ × $^{3}$ + 0.01 × $^{2}$–0.19× | 0.91 | 0.0001 |
| *In vivo* $ADC_{CF}$ (%) | 62.50$^{a}$ | 70.00$^{b}$ | 84.93$^{c}$ | 91.37$^{d}$ | 90.70$^{d}$ | 0.284 | 62.252–7.751*10$^{-5}$ × $^{3}$ + 0.009 × $^{2}$ + 0.178× | 0.99 | 0.0001 |
| *In vivo* $ADC_{GE}$ (%) | 68.47$^{a}$ | 73.70$^{b}$ | 84.70$^{c}$ | 91.51$^{e}$ | 89.77$^{d}$ | 0.126 | 68.390–7.698*10$^{-5}$ × $^{3}$ + 0.009 × $^{2}$ + 0.036× | 0.99 | 0.0001 |

[1] Standard Error of Mean.
[2] Different superscripts within a row (one-way ANOVA comparison) indicate significant differences at $p > 0.05$.

### 3.5. Digestive enzyme activities

The digestive enzyme activities (U mg$^{-1}$) of alkaline protease (2.43–8.50), lipase (4.60–9.50) and amylase (4.67–9.27) in the juvenile crayfish fed the diets containing 25–100% fishmeal replaced with chlorella meal were significantly ($p < 0.05$) higher than those of fed with control diet (Table 4). The significantly highest ($p < 0.05$) activities of alkaline protease, lipase and amylase were measured in crayfish fed the 75%- chlorella meal diet (Table 4). The activities of alkaline protease ($r^2 = 0.97$), lipase ($r^2 = 0.98$) and amylase ($r^2 = 0.97$) increased significantly ($p < 0.05$) as a third-order polynomial response to increased fishmeal substitution levels with chlorella meal in the crayfish diet (Table 4).

### 3.6. Microbiological analysis

Total aerobic bacteria count and fungi count in the intestine of crayfish fed the control diet and the diets containing different levels (25–100%) of fishmeal substituted with chlorella meal did not show any significant difference ($p > 0.05$) (Table 5). As shown in Table 5, with an increment in fishmeal substitution levels with chlorella meal from 25 to 100%, lactobacillus count increased significantly ($p < 0.05$) from 1.48 to 3.80 Log CFU g$^{-1}$ in the intestine of crayfish compared to that of control diet (0.74 Log CFU g$^{-1}$). Although, *E. coli* count reversely decreased ($p < 0.05$) in the intestine of crayfish with an increment in the levels of fishmeal substituted with chlorella meal (Table 5). The lowest *E. coli* count ($p < 0.05$) was measured in the intestine of crayfish fed the diets of 75–100% fishmeal substituted with chlorella meal (Table 5). The lactobacillus count ($r^2 = 0.99$) and *E. coli* count ($r^2 = 0.97$) showed a third-order polynomial response ($p < 0.05$) to increased fishmeal substitution levels with chlorella meal in the crayfish diet (Table 4).

## 4. Discussion

Microalgae products are considered as promising feed ingredients in aquaculture due to easily cultured, non-toxic origin for consumption, suitable protein/fat unit cost, functional carbohydrates and some biological indices including bioactive peptides, antimicrobial properties and unknown growth factors (Ahmad et al., 2020; Bito et al., 2020). However, the form (intact or processed), size and cell wall structure of microalgae are main bottlenecks of using microalgae in the aquatic diet production industry. During the evaluation of feed ingredients, it is important to note suitable palatability, acceptable apparent digestibility coefficients (ADCs) of nutrients, convenient growth performance *via* the retention of nutrients, and finally, maintaining and improving the health of aquatic species during rearing conditions (Glencross et al., 2007; Safari et al., 2014d). One of the strategies to achieve sustainable production of aquatic species is to pay attention to the formulation of diets and diet production methods (*e.g.* hot/cold pelleting, expanding and extrusion) (Nazari et al., 2018; Safari et al., 2014d) and to reduce reliance on the use of fishmeal as a major animal protein source in the diet.

The results of present study showed that partially fishmeal replacement with chlorella meal in the diet of crayfish improved the growth indices including final weight, SGR, FCR, survival rate, and the nutritional indices (PER and PPV). Based on the broken line regression model, the most efficient fishmeal substitution level with chlorella meal was between 78.13% and 85.49% (approximately equal to 320.2–350.5 g kg$^{-1}$ chlorella meal) to reach the maximum SGR value (2.24% body weight day$^{-1}$) and weight gain value (300%), and the minimum FCR value (1.49). Dietary supplementation of 125 g kg$^{-1}$*C. vulgaris* improved the growth performance and survival in the postlarvae of *M. rosenbergii* (Radhakrishnan et al., 2015). Using 20–80 g kg$^{-1}$*C. vulgaris* increased the growth performance, immunity and the survival in the *A. hydrophila*-infected postlarvae of *M. rosenbergii* (Maliwat et al., 2017). Using 100–150 g kg$^{-1}$ chlorella meal in the diet of *P. olivaceus* boosted the antioxidant enzyme activity, lipid metabolism and as a result, growth performance (Rahimnejad et al., 2017). The improvement of growth performance and the nutritional indices of finfish and shellfish species fed the diets containing chlorella meal can be correlated with high nutritive value, namely high crude protein and essential amino acids contents, pigments, vitamins and minerals, of this marine microalgae. It has been previously confirmed that there was a correlation between whole-body chemical composition (crude protein and crude lipid) of experimental aquatic species with nutrient retention rate in muscle (*e.g.*

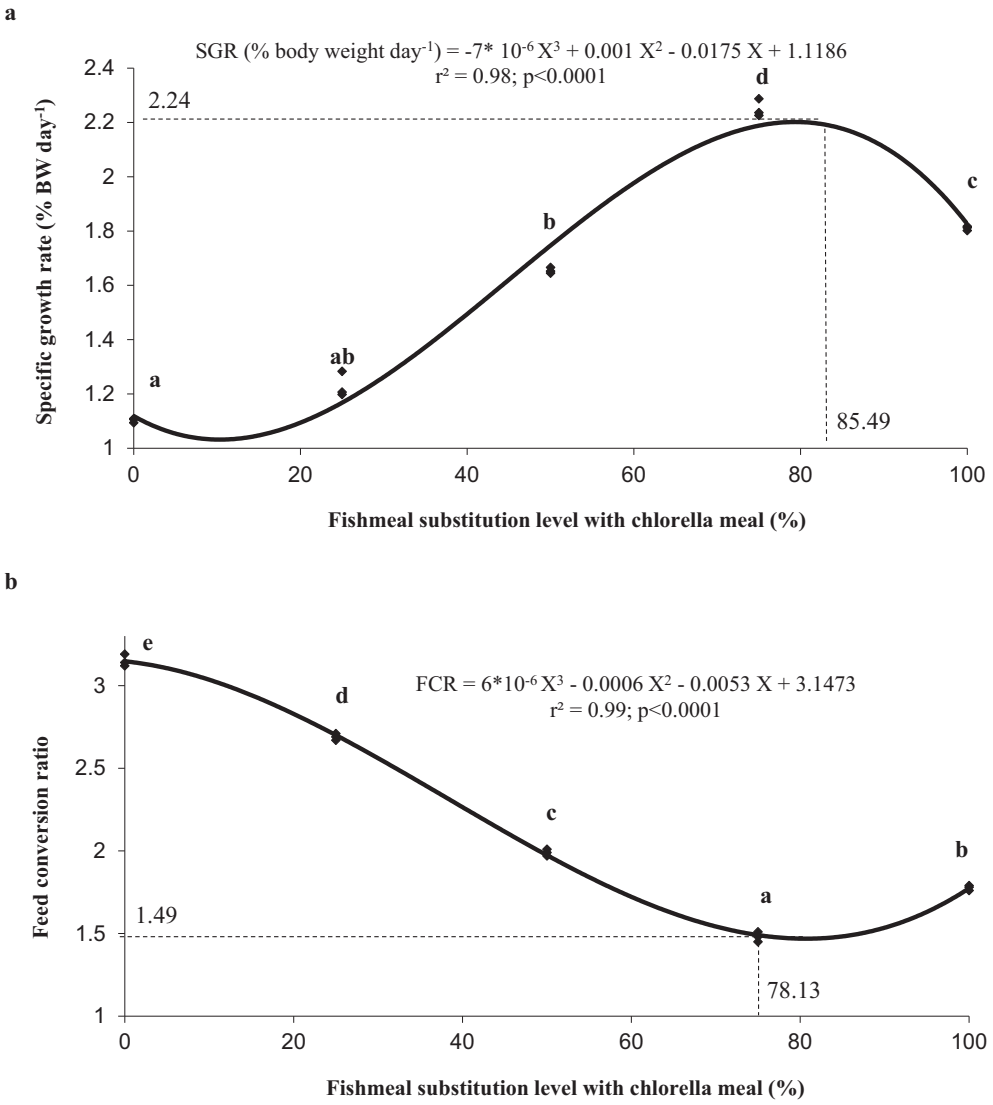


**Fig. 1.** Estimation of dietary fishmeal replacement levels with *C. vulgaris* meal for crayfish (*P. leptodactylus*) by the means of broken line regression analysis of (a) specific growth rate (% body weight (BW) day$^{-1}$) and (b) feed conversion ratio ($n = 3$; Each replicate was stocked with 22 crayfish; $p < 0.05$).

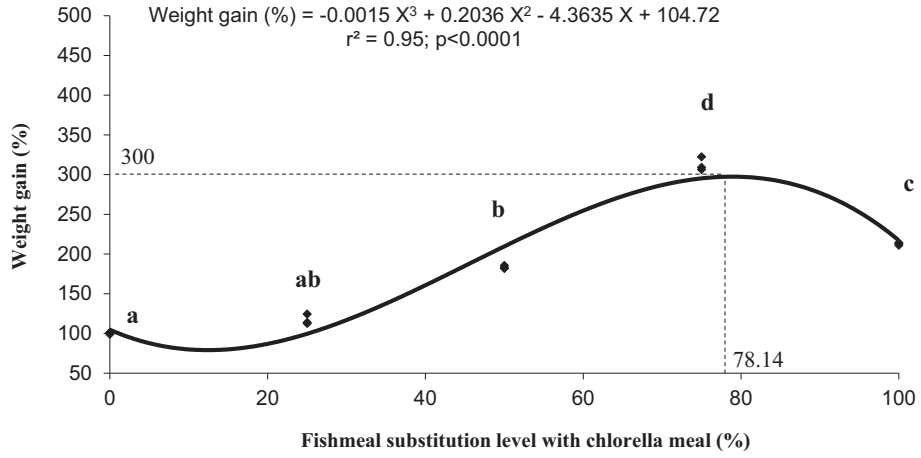


**Fig. 2.** Estimation of dietary fishmeal replacement levels with *C. vulgaris* meal for crayfish (*P. leptodactylus*) by the means of broken line regression analysis of weight gain (%)(n = 3; Each replicate was stocked with 22 crayfish; $p < 0.05$).

**Table 3**
The mean (± SEM[1]) of total haemocyte count (THC, $\times 10^5$ cell $ml^{-1}$), hyaline count (HC, $\times 10^5$ cell $ml^{-1}$), semi-granular count (SGC, $\times 10^5$ cell $ml^{-1}$), large-granular count (LGC, $\times 10^5$ cell $ml^{-1}$), phenoloxidase activity (PO, U $min^{-1}$), superoxide dismutase activity (SOD, U $min^{-1}$), lysozyme activity (LYZ, U $min^{-1}$) and nitric oxide synthase activity (NOS, U $min^{-1}$) of crayfish fed the experimental diets with 0% (control), 25%, 50%, 75% and 100% substitution of *C. vulgaris* meal with fishmeal after 63 days ($n = 3$; Each replicate was stocked with 22 crayfish; $p < 0.05$)[2].

| | Control | Fishmeal replacement levels with *C. vulgaris* in diet (%) | | | | Pooled SEM | Regression analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 75 | 100 | | Regression formula | Adj. $r^2$ | P-value |
| Total haemocyte count | $107.00^a$ | $123.33^b$ | $144.67^c$ | $183.33^e$ | $180.00^d$ | 0.966 | $108.025 + 0.001 \times {}^3 + 0.035 \times {}^2$–0.272× | 0.98 | 0.0001 |
| Hyaline count | $68.67^a$ | $77.00^b$ | $92.00^c$ | $110.00^e$ | $103.00^d$ | 0.830 | $69.014 + 0.0001 \times {}^3 + 0.022 \times {}^2$–0.185× | 0.98 | 0.0001 |
| Semi-granular count | $18.33^a$ | $24.00^b$ | $35.67^c$ | $49.00^e$ | $45.00^d$ | 0.615 | $18.543 + 0.0001 \times {}^3 + 0.017 \times {}^2$–0.154× | 0.99 | 0.0001 |
| Large-granular count | $16.67^a$ | $29.67^b$ | $46.67^c$ | $54.00^d$ | $48.00^c$ | 0.816 | $16.524$–$9.244*10^{-5} \times {}^3 + 0.008 \times {}^2 + 0.396\times$ | 0.99 | 0.0001 |
| Phenoloxidase activity | $1.83^a$ | $2.70^b$ | $4.50^c$ | $7.33^e$ | $6.60^d$ | 0.099 | $1.900$–$2.400*10^{-5} \times {}^3 + 0.003 \times {}^2$–0.48× | 0.98 | 0.0001 |
| Superoxide dismutase activity | $1.50^a$ | $2.70^b$ | $4.20^c$ | $5.40^d$ | $5.23^d$ | 0.116 | $1.507$–$8.889*10^{-6} \times {}^3 + 0.001 \times {}^2 + 0.028\times$ | 0.99 | 0.0001 |
| Lysozyme activity | $2.53^a$ | $3.60^b$ | $4.93^c$ | $9.10^d$ | $9.23^d$ | 0.097 | $2.668$–$2.293*10^{-5} \times {}^3 + 0.004 \times {}^2$–0.059× | 0.96 | 0.0001 |
| Nitric oxide synthase activity | $1.40^a$ | $2.50^b$ | $4.37^c$ | $7.57^e$ | $6.80^d$ | 0.071 | $1.484$–$2.524*10^{-5} \times {}^3 + 0.004 \times {}^2$–0.45× | 0.98 | 0.0001 |

[1] Standard Error of Mean.
[2] Different superscripts within a row (one-way ANOVA comparison) indicate significant differences at $p > 0.05$.

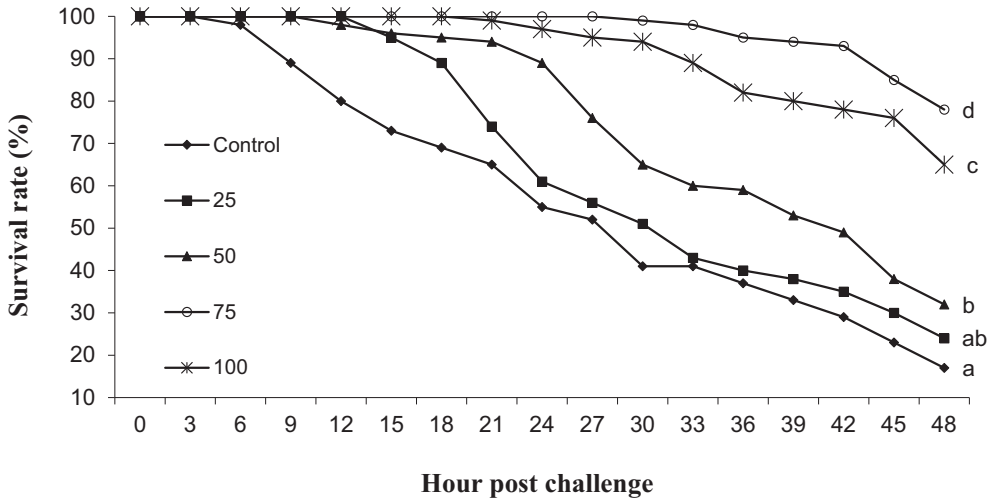


**Fig. 3.** Survival rate (%) of injected crayfish with *A. hydrophila* during 48 h post challenge fed with the experimental diets containing different levels of *C. vulgaris* meal (25, 50, 75 and 100%) substituted with fishmeal (n = 3; Each replicate was stocked with 12 crayfish; $p < 0.05$).

**Table 4**
The mean (± SEM[1]) of activities (U $mg^{-1}$) of (a) alkaline protease, (b) lipase and (c) amylase in the intestine of crayfish fed the experimental diets with 0% (control), 25%, 50%, 75% and 100% substitution of *C. vulgaris* meal with fishmeal after 63 days (n = 3; Each replicate was stocked with 22 crayfish; $p < 0.05$)[2].

| | Control | Fishmeal replacement levels with *C. vulgaris* meal in diet (%) | | | | Pooled SEM | Regression analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 75 | 100 | | Regression formula | Adj. $r^2$ | P-value |
| Alkaline protease | $1.47^a$ | $2.43^b$ | $4.40^c$ | $8.50^e$ | $8.10^d$ | 0.032 | $1.578$–$2.933*10^{-5} \times {}^3 + 0.004 \times {}^2$–0.073× | 0.97 | 0.0001 |
| Lipase | $3.17^a$ | $4.60^b$ | $6.40^c$ | $9.50^e$ | $8.70^d$ | 0.024 | $3.254$–$2.276*10^{-5} \times {}^3 + 0.003 \times {}^2$–0.022× | 0.98 | 0.0001 |
| Amylase | $3.30^a$ | $4.67^b$ | $6.47^c$ | $9.27^e$ | $8.27^d$ | 0.098 | $3.377$–$2.258*10^{-5} \times {}^3 + 0.003 \times {}^2$–0.021× | 0.97 | 0.0001 |

[1] Standard Error of Mean.
[2] Different superscripts within a row (one-way ANOVA comparison) indicate significant differences at $p > 0.05$.

PER and PPV), as well as the growth indices of aquatic species (*e.g.* SGR, FCR, survival rate) (Bito et al., 2020; Govindasamy et al., 2012; Maliwat et al., 2017; Pakravan et al., 2018; Radhakrishnan et al., 2014; Rahimnejad et al., 2017). In order to interpret the results of studies on the fishmeal replacement with various other protein sources in aquafeed aiming the repeatability of results, it is very important to pay attention to type of species, feeding regime (carnivore, herbivore and omnivore), initial weight, nutritional history, use or not use of supplements (*e.g.* limiting amino acids, enzymes and appetizer), control diet formulation and diet processing techniques.

In the present study, the inclusion of chlorella meal in the diet of juvenile crayfish promoted the *in vivo* $ADC_{OM}$, $ADC_{CP}$, $ADC_{CF}$ and $ADC_{GE}$ as well as the activities of alkaline protease, lipase and amylase. We used the intact form of chlorella meal without processing in the present study. The cell digestibility of chlorella was reported as a main problem during including in aquatic diets. The *in vitro* digestion of chlorella cell wall with a combination of digestive enzymes such as trypsin, laminarinase, lytidase, phospholipase, sulfatase, glucanase and lysozyme increased slightly the hydrolysis rate (Gerken et al., 2013). However, the highest digestion rate of chlorella cell wall was accomplished with combination of lysozyme and sulfatase. The findings of present study were not consistence with previous studies. It can be related to be the digestive potential of crayfish to hydrolyze chlorella cell wall. This is likely related to omnivorous feeding regime of crayfish. The potential of *in vitro/in vivo*

**Table 5**
The mean (± SEM[1]) of total aerobic bacteria count (TAB; Log CFU $g^{-1}$), lactobacillus count (LAB; Log CFU $g^{-1}$), *E. coli* count (Log CFU $g^{-1}$) and fungi count (Log CFU $g^{-1}$) of intestine extracted from juvenile crayfish (*P. leptodactylus*) fed on experimental diets with 0% (control), 25%, 50%, 75% and 100% substitution of *C. vulgaris* meal with fishmeal for 63 days (n = 3; Each replicate was stocked with 22 crayfish; $p < 0.05$)[2].

| | Control | Fishmeal replacement levels with *C. vulgaris* meal in diet (%) | | | | Pooled SEM | Regression analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 75 | 100 | | Regression formula | Adj. $r^2$ | P-value |
| Total aerobic bacteria count (Log CFU $g^{-1}$) | 6.29[a] | 6.27[a] | 6.29[a] | 6.28[a] | 6.28[a] | 0.004 | | | |
| Lactobacillus count (Log CFU $g^{-1}$) | 0.74[a] | 1.48[b] | 2.41[c] | 3.80[e] | 3.66[d] | 0.012 | 0.768–9.138*$10^{-6}$ × $^{3}$ + 0.001 × $^{2}$–0.002× | 0.99 | 0.0001 |
| *E. coli* count (Log CFU $g^{-1}$) | 1.32[d] | 1.18[c] | 1.04[b] | 0.75[a] | 0.76[a] | 0.009 | 1.315 + 1.600*$10^{-6}$ × $^{3}$ + 0.0001 × $^{2}$ + 0.0001× | 0.97 | 0.0001 |
| Fungi count (Log CFU $g^{-1}$) | 0.54[a] | 0.55[a] | 0.54[a] | 0.54[a] | 0.54[a] | 0.005 | | | |

[1] Standard Error of Mean.
[2] Different superscripts within a row (one-way ANOVA comparison) indicate significant differences at $p > 0.05$.

digestion in crayfish can confirmed that this species has omnivorous behavior (Safari et al., 2014b). It is interesting that 10% fishmeal substitution level with *C. vulgaris* meal (97.2 g $kg^{-1}$) in the diet of L. *vannamei* increased the activities of trypsin and amylase (Pakravan et al., 2018). Trypsin activity increased due to feeding mitten lobster, *Parilibacus japonicas* with the diet containing microalgae (Le Vay et al., 1993). Although, it is not clear the relation between digestive enzyme activities and feeding the aquatic species with microalgae. Some factors such as high quality of protein in marine microalgae and existing free amino acids can be considered as main factors affecting on physiological pathways to activate digestive enzymes (James et al., 2006; Le Vay et al., 1993; Pakravan et al., 2017). However, it needs further investigations in future.

The results of present study showed that feeding juvenile crayfish with the chlorella supplemented- diets increased lactobacillus count and decreased *E. coli* count. Some authors suggested that microalgae-supplemented diets boosted the benign microbes in the gastrointestinal tract in order to produce more endogenous digestive enzymes (Anand et al., 2013; Reitan et al., 1993). Consistent with our results, feeding red swordtail, *Xiphophorus helleri*, with spirulina supplemented -diets improved the growth performance, fertility, coloration and intestinal flora (James et al., 2006). Increasing in colony forming unit of biota in the gastrointestinal tract led to breakdown the consumed feed to more digestible nutrients (James et al., 2006; Safari et al., 2017). In the present study, it seems that the difference between *in vivo* apparent digestible coefficients of nutrients (organic matter, crude protein and crude fat) and gross energy of experimental diets containing chlorella meal with the control diet can be attributed to the presence of beneficial biota. It is likely attributed to match the carbohydrate content existing in chlorella meal as a prebiotic substrate with beneficial bacteria existing in the digestive tract of crayfish. It has been confirmed that dietary supplementation with the potential feed additives such as probiotics, prebiotics and synbiotics can boost the disease resistance *via* some inhibiting pathways in the digestive tract like competition for binding site in the digestive tract, pH reduction, the secretion of organic acids and antibiotics from beneficial microflora (Li et al., 2007; Manning and Gibson, 2004). That is why it is recommended to design *in vitro* experiments in order to select the best synbiotic between chlorella meal and useful bacteria in future studies.

In the present study, feeding juvenile crayfish with the diets containing different levels of fishmeal substituted with chlorella meal showed positive effects on the hemolymph indices (THC, HC, SGC and LGC), antioxidant enzymes (PO, SOD and NOS), immune responses (LYZ) and, also promoted resistance against a biological stressor, *viz.* injection with *A. hydrophila*. Hemocyte counts (THC, HC, SGC and LGC) play key roles in the wellbeing of crustacean species (Jussila et al., 1997) through cytotoxicity and the control of physiological pathways of the prophenoloxidase system (Johansson et al., 2000). It has been confirmed that short chain fatty acids including acetic, propionic, and specially butyrate acids as final products of synbiotic products (Safari and Paolucci, 2018) have positive effects on growth performance, immunity and survival rate of some aquatic species including crayfish (*P. leptodactylys*), rainbow trout (*Oncorhynchus mykiss*), kutum (*Rutilus frisii*), angelfish (*Pterophyllum scalare*) and zebrafish (*Danio rerio*) (Cummings and Macfarlane, 2002; Hoseinifar et al., 2015; Maslowski and Mackay, 2011; Scheppach, 1994; Schley and Field, 2002). Butyrate as a main energy source for gastrointestinal microvilli (Maslowski and Mackay, 2011) can enhance *in vivo* apparent digestibility coefficients of nutrients and gross energy (Safari et al., 2014c; Ye et al., 2011), develop nutrient efficiency indices (*e.g.* FCR) (Buentello et al., 2010), activate the physiological pathways of immunity and, finally protect host on stressors (Maslowski and Mackay, 2011; Safari and Paolucci, 2017b). Short chain fatty acids can boost lipid synthesis and maybe, enhance the metabolic pathways related to nutrient retention (Marcil et al., 2002). It has been confirmed that butyrate can down-regulate the expression of *Salmonella sp.* as an invasion gene (Van Immerseel et al., 2006). It appears clearly that the supplementation of aquatic diets with marine microalgae can be used for two purposes including nutrient supply (*e.g.* crude protein, crude fat, vitamins and minerals) and functional properties. Overall, it seems that the reason for the improvement of immune responses, antioxidant enzyme activities and hemolymph indices in crayfish fed the diets with β-1,3-glucan existing in chlorella meal could be due to the interaction between the beneficial intestinal bacterial flora and the chlorella cell wall as a synbiotic. This can increase the activity of beneficial bacteria in the gastrointestinal tract and ultimately, led to produce organic acids in the intestine. However, these cascading responses need to be explained *via* physiological models in future studies. It has been confirmed that organic acids have positive effects on the diet quality (reduction of pH and harmful bacteria intake), stomach functions (increment in enzyme activity and mineral solubility), intestine performance (increment in nutrient digestibility, mineral availability and gut health), feces traits (reduction of phosphorus load and microbiota count) and finally, the improvement of water quality (Ng and Koh, 2017; Safari et al., 2020). One of the future prospects of the aquafeed production industry is to focus on how to use bioactive peptides originated from various feed ingredient such as marine microalgae in the diet. However, this issue merits further investigations.

In this trial, the chlorella supplemented diets increased the survival rate of *A. hydrophila*- injected crayfish. Innate immunity is the most important barrier against any biological pollution in decapods (Zhang et al., 2011) *via* hemolymph migration to the injection site and the lysis by hemocyte (Sang et al., 2009). Triple production rate of hemocyte in the lipopolysaccharide- injected kuruma prawns was reported in literature (Sequeira et al., 1996). Using some synbiotics including *Enterococcus faecalis* + galactooligosaccharide or *Pediococcus acidilactici* + galactooligosaccharide (Safari and Paolucci, 2017b) and some prebiotics (mannan-oligosaccharide and fructo-oligosaccharide) (Safari et al., 2014a) in the diet of crayfish, *P. leptodactylus,* improved the growth performance, immune responses and *A. hydrophila* expsosure challenge. Dietary supplementation of black tiger shrimp, *Penaeus*

*monodon* with β-1,3-glucan enhanced hemocyte phagocytic activity and superoxide anion production and decreased clotting time (Chang et al., 2000) and also, improved immune system against white spot syndrome virus (Chang et al., 2003). Improving in the survival rate of injected crayfish fed the chlorella diets in the present study is likely attributed to be some functional compounds in chlorella meal (*e.g.* free amino acids and fatty acids, pigments, minerals, and vitamins). A wide range of these functional (nutraceutical) compounds in the marine micro- and macro algae have been reported in the literature (Ahmad et al., 2020; Bito et al., 2020). However, further studies need to determine the best forms of using chlorella products (meal, protein concentrate and isolate), inclusion level, and diet preparation techniques.

## 5. Conclusion

In the current trial, juvenile crayfish fed the diets containing 75% chlorella meal substituted with fishmeal exhibited the highest values of growth indices (final weight, SGR and survival rate) and nutritional efficiency indices (PER and PPV) and the lowest FCR value. The juvenile crayfish fed the chlorella meal- diet showed higher hemolymph indices (THC, HC, SGC and LGC) than those of fed the control diet. The results indicated that chlorella meal had the potential to promote juvenile crayfish immune responses of *P. leptodactylus* against *A. hydrophila* injection. Finally, based on the results of present study, the objectives of the work were achieved and the inclusion of chlorella meal in the diet of crayfish can be recommended.

## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Animal welfare and feed legislation

The authors declare that experiments were done according to FUM animal ethics and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

## Authors' contributions

Omid Safari: Conceptualization, Funding acquisition, Formal analysis.Hamidreza Ahmadniaye Motlagh: Project administration. Marina Paolucci: Validation, Writing, Editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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