



Effect of dietary supplementation of crude microalgal extracts on growth performance, survival and disease resistance of *Lates calcarifer* (Bloch, 1790) larvae

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ABSTRACT

The present study aimed to assess growth performance and health status of *Lates calcarifer* larvae fed with crude microalgal extracts. Larvae weighing 5.96 ± 0.39 mg were randomly divided into four experimental groups in triplicates, with each replicate having 35 larvae. Four diets formulated to contain 55% protein and 12% lipid, were prepared with pigment rich microalgal extracts at 0% (T1) and 1% each of crude phycocyanin (T2), chlorophyll (T3) and astaxanthin (T4) extracts. The experiment was carried out in 30 l plastic tanks by feeding experimental diets to satiation. There was no significant difference ($p < 0.05$) in growth performance in larval groups fed different experimental diets. Percentage survival during the feeding experiment was significantly higher in the astaxanthin fed group (77.14 ± 1.65) and lower in chlorophyll fed group (36.19 ± 0.95) compared to control group. Cannibalism (%) was significantly reduced in the treatments fed astaxanthin (7.61 ± 0.95) and phycocyanin (8.57 ± 0.3) pigmented diets compared to chlorophyll fed group (13.33 ± 0.95) and control group. Bacterial challenge with pathogenic *Vibrio alginolyticus* revealed highest resistance in astaxanthin and phycocyanin fed larvae. The study confirms that astaxanthin and phycocyanin at 1% dietary levels improve survival and disease resistance in Asian seabass larvae but do not affect growth performance.

Keywords: Astaxanthin, Chlorophyll, Larval feed, *Lates calcarifer*, Microdiets, Phycocyanin

Introduction

Fish larvae are vulnerable during the first stages of development and are highly responsive to biotic and abiotic conditions. Asian seabass, *Lates calcarifer* (Bloch, 1790) has altricial ontogenetic development and predatory feeding habit which increases occurrence of coeval cannibalism. The cause of cannibalism is a conglomerate of genetic and behavioural factors and poorly acceptable feeds may enhance, or even trigger cannibalism (Hamre *et al.*, 2013). Mortality is more frequent in the first two months after hatching. The weaning of marine fish larvae towards formulated microdiets is important to simplify the rearing procedures and reduce mortality. Although a number of studies have evaluated the survival and health with respect to various factors like growth rate and stocking density (Barlow *et al.*, 1995; Kailasam *et al.*, 2002; Kailasam *et al.*, 2007), the effect of dietary additives remains largely unknown.

Algae are a group of nonvascular autotrophic organisms that are able to produce a wide range of

pigments and bioactive compounds that can be used in aquaculture diets (Spolaore *et al.*, 2006). Astaxanthin is a secondary carotenoid that finds use in aquaculture. *Haematococcus pluvialis* synthesises astaxanthin in its cysts and has been studied in detail as a commercial source of astaxanthin (Guerin *et al.*, 2003). Phycocyanin is a phycobiliprotein and the major photosynthetic pigment in cyanobacteria (Patil and Raghavarao, 2007). Phycocyanin is not only used as a colouring agent (Yoshida *et al.*, 1996) but also as a therapeutic agent against oxidative stress (Bhat and Madyastha, 2001). Chlorophyll (chl) *a*, is a blue green pigment which, unlike chl *b* and *c*, is found in all photosynthetic organisms. Chlorophyll *a* has been known for its bioactive properties (Dashwood *et al.*, 1998; Simonich *et al.*, 2008). Pigments improve the welfare of aquatic animals during the larval stages. This is mostly reflected in the improvement of survival of larvae (Vassallo-Agius *et al.*, 2001; Chuchird *et al.*, 2015) in culture settings. However, studies evaluating the direct effect of dietary microalgal pigments on the welfare of fish larvae are scarce. Therefore, present study was conducted

with the objective to evaluate the growth performance, survival, stress resistance and disease resistance of seabass larvae weaned onto microdiets supplemented with crude microalgal extracts. Knowledge of the effect of crude microalgal extracts on these parameters may help to improve survival rates in captive rearing.

Material and methods

Feed pigments and pigment extraction

Three different types of natural pigments were used to provide colour to the feed particles. Phycocyanin rich extract, from *Spirulina platensis*, is dark blue in colour and provides green colour to the feed. Astaxanthin, from *H. pluvialis*, is red in colour and imparts red colour to the feed. Chlorophyll rich extract, from *Chlorella*, is blackish green in colour and provides black colour to the feed.

Phycocyanin was extracted from freeze-dried *S. platensis* (Ocean Nutrition International LLC, USA). The phycocyanin pigment was extracted from the algae by freeze thaw method (Sivasankari *et al.*, 2014). The content of phycocyanin, which could be extracted in the crude extract, was calculated by using the formula (Bennett and Bogorad, 1973):

$$PC \text{ (mg ml}^{-1}\text{)} = \frac{A_{615} - 0.474 (A_{652})}{5.34}$$

where PC = Phycocyanin content of the extract (mg ml⁻¹); A₆₁₅ = Absorbance at 615 nm; A₆₅₂ = Absorbance at 652 nm.

Table 1. Composition of experimental diets [g%]

Ingredients	T1	T2	T3	T4
Fish meal	54	54	54	54
Acetes meal	14	14	14	14
Soyabean meal	4	4	4	4
Squid meal	5	5	5	5
Wheat gluten	10	10	10	10
Fish oil	2	2	2	2
Lecithin	4	4	4	4
Vitamin-mineral mix	2.5	2.5	2.5	2.5
Yeast	3	3	3	3
Vitamin C	0.3	0.3	0.3	0.3
Vitamin E	0.2	0.2	0.2	0.2
Pigment/Wheat flour	1	1	1	1
Total	100	100	100	100
Proximate composition (g%)				
Moisture	8.75 ± 0.88	8.20 ± 0.56	8.45 ± 0.33	8.34 ± 0.93
In dry matter				
Crude protein	55.19 ± 1.4	55.23 ± 1.3	54.97 ± 1.7	55.34 ± 0.9
Crude lipid	12.23 ± 0.34	12.15 ± .56	11.98 ± 0.22	12.21 ± 0.12
Ash	16.19 ± 0.21	16.45 ± 1.3	17.32±0.4	16.54 ± 0.9
Crude fiber	2.49 ± 0.12	2.43 ± 0.22	2.65 ± 0.43	2.21 ± 0.34

Composition of Vitamin-mineral mix (PREMIX PLUS) (quantity per 2.5 kg): Vitamin A - 5 500 000 IU; Vitamin D3 - 1 100 000 IU; Vitamin B2 - 2000 mg; Vitamin E - 750 mg; Vitamin K - 1000 mg; Vitamin B6 - 1000 mg; Vitamin B12 - 6 µg; Calcium pantothenate - 2500 mg; Nicotinamide - 10 g; Choline chloride - 150 g; Mn - 27 000 mg; I - 1000 mg; Fe - 7500 mg; Zn - 5000 mg; Cu - 2000 mg; Co - 450 mg; L-lysine - 10 g; DL-Methionine - 10 g; Selenium - 50 ppm

The liquid crude extract obtained was dried in a hot air oven at 40°C for 24 h in a petridish yielding the solidified extract that was scraped carefully from the petridish to obtain crude phycocyanin flakes. The flakes were ground in a mixer to obtain fine powder and used for feed preparation.

The chlorophyll rich crude extract was obtained from freeze-dried *Chlorella* (Ocean Nutrition International LLC, USA). It was extracted using acetone as the solvent (Ilavarasi *et al.*, 2012) and the crude extract was then concentrated in a rotary evaporator. The chlorophyll content in the crude extract was calculated using the formula (APHA, 2012):

$$\text{Chlorophyll (mg m}^{-3}\text{)} = \frac{26.7[(\text{OD}_{664} - \text{OD}_{750}) - (\text{OD}_{665} - \text{OD}_{750})] \cdot V_1}{V_2 \times L}$$

where V₁ = Volume of the extract (L); V₂ = Volume of the sample (m³); L = Length of the cuvette (cm); OD₆₆₄ = Absorbance at 664 nm; OD₇₅₀ = Absorbance at 750 nm; OD₆₆₅ = Absorbance at 665 nm

Carophyll Pink™ (5% pure) extracted from *H. pluvialis* (Ocean Nutrition International LLC, USA) was used as astaxanthin source.

Experimental diets

Four different types of experimental diets having crude protein 55% and crude lipid 12% were prepared (Table 1): T1: No pigment; T2: 1% Phycocyanin; T3: 1%

Chlorophyll and T4: 1% Astaxanthin. For each treatment, diets were prepared by micro extrusion marumerisation technique (Barrows and Lellis, 2006). After carefully weighing the ingredients, they were thoroughly mixed in plastic buckets. Pigments were then added and the ingredients were again thoroughly mixed. This was followed by the addition of water to form a mash. The ingredient mash was then divided into two parts by weight. One part was cold extruded in 300 μ mesh, at 15 revolutions per min each. The noodles obtained were then subjected to marumerisation in a cylindrical chamber with a rotating bottom plate. The bottom plate was rotated at a speed of 800 rotations per min for 90 s to obtain microdiets of 300 μ m particle size. The second part of the mash was cold extruded in a 500 μ m mesh and then subjected to marumerisation, with conditions being the same as for the first part of the mash. This yielded microdiets of 500 μ m particle size. The microdiets were carefully kept to dry in hot air oven at 40°C for 1 h and stored in zip-lock covers until use. The proximate analysis for crude protein (Kjeldahl nitrogen \times 6.25), total fat (ether extraction), ash (residue after 4 h at 600°C), moisture (weight loss after drying at 105°C 4-5 h) and crude fibre (acid-alkali wash) was carried out following standard methods (AOAC, 1995).

Experimental fish and feeding

Four hundred and twenty *L. calcarifer* larvae at 16 days post-hatch (dph) were used for the feeding experiment. Thirty-five larvae were randomly stocked in 12 tubs (four treatments having three replicates each) of volume 30 l each. Co-feeding of *Artemia* nauplii and experimental microdiets was continued until the larvae were completely weaned to experimental microdiets on 21 dph. The feeding was continued for 30 days (16 to 46 dph). Water quality parameters were maintained optimally.

Survival study and growth performance

The following formulae were used to evaluate the survival and growth performance of the larvae:

$$\text{Weight gain (\%)} = 100 \times (\text{Average final weight} - \text{Average initial weight}) / \text{Average initial weight}$$

$$\text{Specific growth rate (\% day}^{-1}\text{)} = [(\ln \text{Average final weight} - \ln \text{Average initial weight}) / \text{Time (days)}] \times 100$$

$$\text{Cannibalism (\%)} = 100 \times \{ \text{Initial number of fish} - (\text{Final number of fish} + \text{Number of dead fish registered}) \} / \text{Initial number of fish}$$

$$\text{Coefficient of variation (\%)} = (\text{Standard deviation of individual weight} / \text{Mean individual weight}) \times 100$$

$$\text{Size heterogeneity (weight)} = \text{Final coefficient of variation} / \text{Initial coefficient of variation}$$

$$\text{Survival rate (\%)} = (\text{Final number of fish} \times 100) / \text{Initial number of fish}$$

Fulton's condition equation was used to find out the condition factor:

$$K = (W \times 10^5) / TL^3$$

where K is the condition factor, W is weight (g) and TL is average total length (mm).

Antioxidant enzymes activities

On termination of the feeding experiment, three larvae from each tank were weighed and homogenised in phosphate buffered saline (PBS) solution with a pH of 7.6, kept in an ice-water bath. After centrifugation (13,000 g, 5°C, 15 min), the supernatant was used for determination of enzyme activities. Superoxide dismutase (Superoxide: Superoxide oxidoreductase, EC 1.15.1.1) was assayed in the homogenate by the method of Marklund and Marklund (1974). The enzyme activity was expressed as units per mg of protein in tissues. The reaction was initiated by the addition of 0.5 ml pyrogallol reagent and the change in optical density was measured at 480 nm for 3 min. Fifty percent inhibition of pyrogallol by the enzyme was taken as one enzyme unit. Catalase (Hydrogen peroxide: Hydrogen-peroxide oxidoreductase, EC 1.11.1.6) activity was assayed by the method of Sinha (1972). Absorbance was read at 570 nm. Catalase activity was expressed as micromoles of H₂O₂ consumed per minute per mg of protein.

Bacterial challenge study

For determination of LC₅₀ value, purified *Vibrio alginolyticus* culture from glycerol stock was revived in Zobell-Marine broth. The bacteria were plated on thiosulphate citrate bile salt sucrose (TCBS) agar by the spread-plate technique to obtain single colony. A single colony was taken and again regrown in tryptic soy broth (TSB) supplemented with 2% NaCl. The culture broth was centrifuged at 3000 g for 10 min. The pellets were suspended in sterile saline (0.9% NaCl, pH 7.4) and the OD of the solution was adjusted to 0.6. This corresponded to a count of 1.40 \times 10⁸ cfu ml⁻¹, confirmed by total plate count. The bacterial cells were then diluted to get different concentrations required for the experiment. Eighty larvae that were being fed the control experimental diet (Table 1) and were from the same source as the experimental fish were used for LC₅₀ study. They were distributed randomly into 8 groups with 10 larvae each. Each of seven groups were bath challenged by bacteria at concentrations of 1.4 \times 10¹ to 1.4 \times 10⁷ cfu ml⁻¹ for 1 h. The control group was maintained in sterile seawater. The larvae were

monitored for 96 h and in case of mortality, the causative agent was confirmed by re-isolating *V. alginolyticus* from the moribund larvae. LC_{50} value was calculated as per the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977).

For the bacterial challenge experiment, 10 larvae from each replicate tank were immersed for 1 h in seawater containing bacterial cells. Larvae were then maintained separately in 10 l tubs of seawater and monitored for mortality for the following 7 days. From the data of the percentage mortality, relative percentage survival (RPS) against the control group was calculated at the end of 7 days of challenge, using the following formula (Ahmad *et al.*, 2016):

$$RPS = 1 - \left(\frac{\text{Percentage mortality in treatment}}{\text{Percentage mortality in control}} \right) \times 100$$

Statistical analysis

To validate the reproducibility of results, each assay was done in triplicate. The results were treated by one-way analysis of variance (ANOVA) using *SPSS v. 16*. Comparison among different treatments was made using Duncan's Multiple Range Test (DMRT). All analyses were performed considering a level of 95% of confidence ($p < 0.05$).

Results

Feed pigments and pigment extraction

The phycocyanin content in the crude extract was 1.3 mg ml^{-1} that corresponded to the yield of 65 mg g^{-1} freeze dried *Spirulina*. The chlorophyll content in the crude extract was $0.0645 \text{ mg ml}^{-1}$ corresponding to a yield of 6.45 mg g^{-1} freeze dried *Chlorella* (Fig. 1).

Experimental diets

The extracted pigments were concentrated and used in the development of diets of different colours. Concentrated pigment extracts were added to the feed at 1% inclusion level (Fig. 2). No pigment was added in control diet (T1), which retained the brown colour of the basal diet. The phycocyanin and chlorophyll pigmented feeds developed green (T2) and black (T3) colour

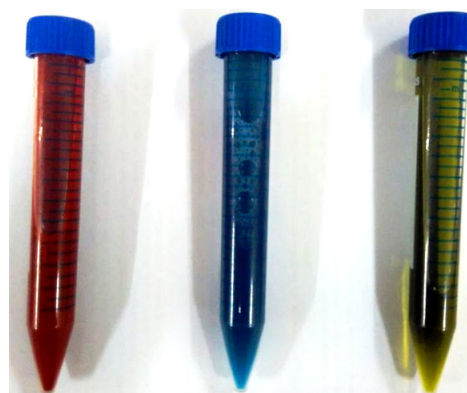


Fig. 1. (a) Crude Astaxanthin, (b) Phycocyanin and (c) Chlorophyll extracts used for diet preparation



Fig. 2. Experimental diets prepared for the feeding trial using different pigments in crude form as additives.

(a) 1% Astaxanthin, (b) 1% Chlorophyll, (c) 1% Phycocyanin and (d) Control diet with no pigment additive

respectively. The astaxanthin based feed developed red colour (T4).

Growth performance, size heterogeneity and condition factor

There was no significant difference ($p < 0.05$) in the percentage weight gain, specific growth rate, daily growth rate, coefficient of variation, size heterogeneity and condition factor in the different experimental groups (Table 2).

Table 2. Weight gain (%), specific growth rate (GR), average daily growth (mg day^{-1}), coefficient of variation (%), size heterogeneity and condition factor (K) of *L. calcarifer* larvae

Treatment	Weight gain (%)	Specific growth rate ($\% \text{day}^{-1}$)	Average daily growth (mg day^{-1})	Coefficient of variation (%)	Size heterogeneity (%)	Condition factor (K)
T1	2308.91±507.47	4.54±0.2	4.497±1.06	61.28±10.23	1.18±0.19	2.64±0.15
T2	1809.59±183.98	4.33±0.13	3.85±0.32	60.91±6.99	1.18±0.13	2.85±0.30
T3	2642.17±699.55	4.59±0.36	4.69±1.27	59.12±4.70	1.14±0.09	2.68±0.20
T4	1959.58±65.58	4.60±0.04	4.46±0.12	53.86±7.64	1.04±0.14	2.39±0.04

Values represent Mean ± SE. Mean values are data of three replicates (n=3). Values in the same column with no significant difference ($p < 0.05$) have no superscript. T1 - Control; T2 - 1% Phycocyanin, T3 - 1% Chlorophyll, T4 - 1% Astaxanthin

Survival and cannibalism

The survival (%) of larvae in the different experimental groups was highest ($p < 0.05$) in the astaxanthin fed groups compared to other groups (Fig. 3). The group fed the chlorophyll pigmented feed exhibited significantly lower survival ($p < 0.05$) than all the other groups. The mortality due to cannibalism over the 30 days feeding experiment was significantly lower in T2 and T4 groups compared to the control group. However, there was no significant difference between T2 and T4 (Fig. 4).

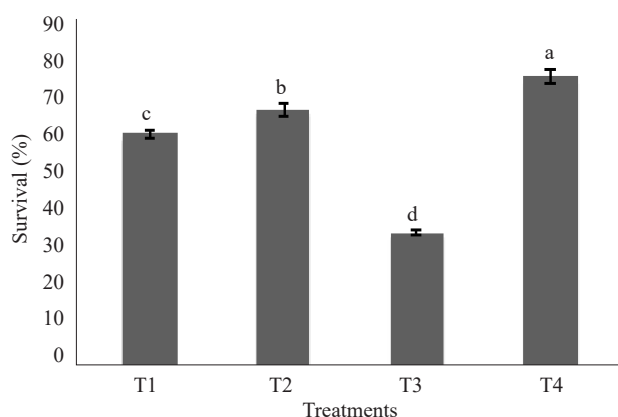


Fig. 3. Percentage survival of different experimental groups. Mean values ($n = 3$) in the experimental group with different superscripts (a, b, c and d) differ significantly ($p < 0.05$). Data expressed as Mean \pm SE. T1 - Control; T2 - 1% Phycocyanin, T3 - 1% Chlorophyll, T4 - 1% Astaxanthin

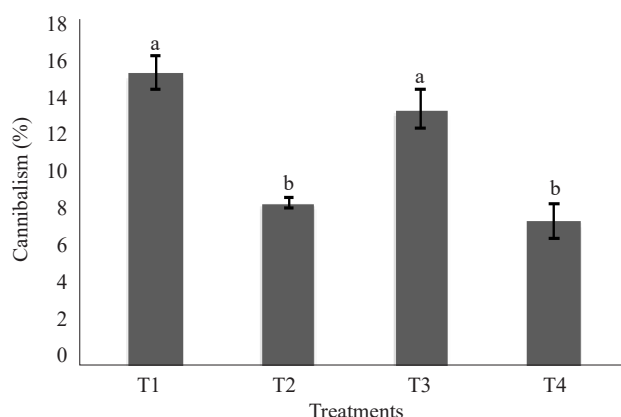


Fig. 4. Mortality due to cannibalism (%) in different experimental groups. Mean values ($n = 3$) in the experimental group with different superscripts (a, b) differ significantly ($p < 0.05$). Data expressed as Mean \pm SE. T1 - Control; T2 - 1% Phycocyanin, T3 - 1% Chlorophyll, T4 - 1% Astaxanthin

Antioxidant enzyme activity

Both superoxide dismutase and catalase enzymes showed significantly reduced activity in the group fed

phycocyanin pigmented feed (T2). The activities of the two enzymes in the other three experimental groups were not significantly different from each other (Fig. 5).

Challenge study

The LD_{50} value obtained after 5 days of observation period for the viable cells of *V. alginolyticus* to fingerlings was found to be $1.4 \times 10^{4.5}$ cfu ml⁻¹. After immersion challenge by the same concentration of bacteria in the experimental fish, the first mortality was observed at 8 h. At the end of the 7 days challenge period, the groups fed phycocyanin (T2) and astaxanthin (T4) pigmented diets exhibited significantly lower mortality ($p < 0.05$). Mortality was highest in the control group but it was not significantly different from the group fed chlorophyll pigmented feed. The relative percentage of survival (RPS) for the treatment groups (T2, T3 and T4) was calculated against control group that was assumed to have 0% survival. The RPS was highest in the group fed the astaxanthin-pigmented diet followed by phycocyanin-pigmented diet (Fig. 6).

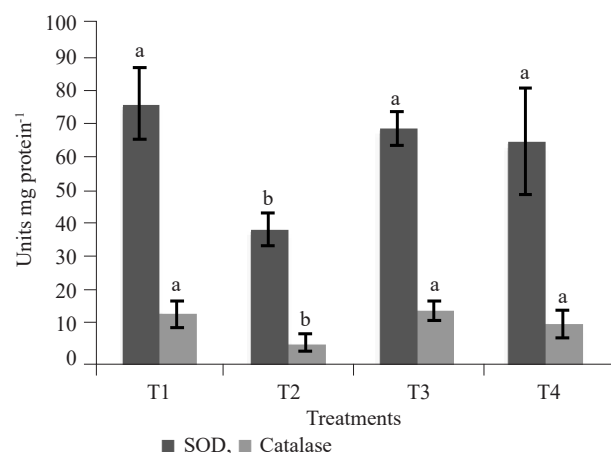


Fig. 5. Status of anti-oxidant enzymes of *L. calcarifer* larvae fed different experimental diets. Mean values ($n = 3$) in the experimental group with different superscripts (a, b) differ significantly ($p < 0.05$). Data expressed as Mean \pm SE. T1 - Control; T2 - 1% Phycocyanin, T3 - 1% Chlorophyll, T4 - 1% Astaxanthin

Discussion

In fish larval nutrition, the microalgae are either used directly or as food of live prey (Brown *et al.*, 1997). Microalgae may also be used to obtain crude extract for use in aquafeeds (Apt and Behrens, 1999). In the present study, effect of microalgal crude extracts on cannibalism, growth performance, survival and disease resistance of seabass larvae was evaluated.

Efficiency of extraction of phycocyanin from *Spirulina* is affected by the cellular disruption method, type of solvent, biomass-solvent ratio and extraction time

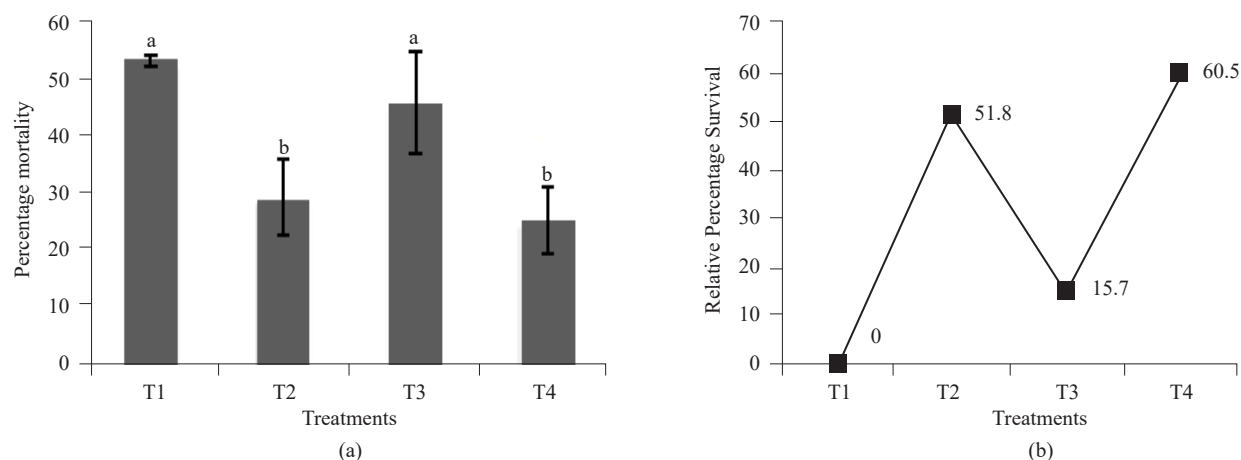


Fig. 6. Percentage mortality (A) and Relative percentage of survival (B) compared to Control group (T1). Mean values (n=3) with different superscripts (a, b) differ significantly ($p < 0.05$) and with the same superscripts do not differ significantly. Data are expressed as Mean \pm SE. T2 - 1% Phycocyanin, T3 - 1% Chlorophyll, T4 - 1% Astaxanthin

(Abalde *et al.*, 1998; Reis *et al.*, 1998). Common methods for extraction and purification of phycocyanin involve break-up of cells using high-pressure homogenisation, sonication, freeze thawing, or enzymatic treatment (Eriksen, 2008). We obtained phycocyanin yield of 65 mg g⁻¹ at a solvent biomass ratio of 1:50 by rapid freezing and thawing method. Dried *Spirulina* has shown lower yield of phycocyanin as compared to its fresh biomass (Sarada *et al.*, 1999). However, we found no such reduction in the yield compared to previous studies that used fresh *Spirulina* (Minkova *et al.*, 2003; Sandeep *et al.*, 2013). Chlorophyll is a lipid soluble compound and is usually extracted in organic solvents (Simon and Helliwell, 1998). Usually acetone is used as the solvent with duration of extraction and number of extraction steps affecting the yield of chlorophyll (Schumann *et al.*, 2005). Cell disruption of *C. vulgaris* by ultrasound bath yielded chlorophyll content of 13.10 $\mu\text{g ml}^{-1}$ in the extract (Seyfabadi *et al.*, 2011). In the present study, instead of ultrasound we used mechanical stirring in acetone, yielding chlorophyll content of 6.45 mg g⁻¹ dry *Chlorella*. The use of astaxanthin in aquaculture has been studied in great detail for decades. Astaxanthin is known as the natural colourant giving some fish and crustaceans their characteristic body colour (Fujii *et al.*, 2010). Although the present study did not focus on the pigmentation of larvae fed with astaxanthin-enriched diet, the same source of astaxanthin (Carophyll PinkTM) was used.

Different studies carried out to evaluate the effect of astaxanthin on growth performance of fish reveal conflicting results (Amar *et al.*, 2001; Seyed *et al.*, 2013). We found no significant difference in the growth performance of different experimental groups. A number of studies suggest that the growth promotion by astaxanthin

is not direct and depends on other extraneous variables (Storebakken and Choubert, 1991; Christiansen *et al.*, 1994). Indeed, it will be interesting to decipher the factors affecting the capacity of astaxanthin to improve growth performance. Although bioactive properties have been associated with both chlorophyll (Simonich *et al.*, 2008) as well as phycocyanin (Hayashi *et al.*, 2006), the growth promoting effects of these bioactive compounds in fish larvae is unknown. The present study represents the first attempt to evaluate the effect of these dietary compounds on the growth performance of seabass larvae. We found no significant effect on the growth performance when the seabass larvae were fed chlorophyll or phycocyanin extracts.

Astaxanthin and phycocyanin fed groups exhibited higher survival and the chlorophyll fed group showed lower survival than the control group. Dietary astaxanthin has been observed to improve the survival of fish in earlier studies (Chuchird *et al.*, 2015). The improvement of survival in the group fed dietary astaxanthin may be attributed to a number of reasons: the provitamin A function of astaxanthin (Christiansen *et al.*, 1994) and immune stimulation (Jyonouchi *et al.*, 1995). Phycocyanin obtained from *S. platensis* has been reported to exhibit antioxidant activity (Soni *et al.*, 2008). Feeding phycocyanin may attribute better survival of larvae through antioxidant and radical scavenging properties of phycocyanin help in reducing the oxidative stress. This is further supported by our observation of reduction in the antioxidant enzyme activities in the treatment group fed phycocyanin pigmented feed. The higher mortality in T3 may be attributed to lipid soluble feed deterrents, that may have been activated or co-extracted along with chlorophyll. Similar feed deterrent compounds have been

observed from other marine autotrophs (Paul, 1987). The presence of these compounds in the feed may have reduced the feeding activity of larvae in T3. As starvation induces cannibalism (Folkvord, 1991), the same treatment group (T3) also reported the highest cannibalism incidences. Therefore, higher cannibalism in T3 supports our assumption that the presence of chlorophyll reduced the palatability of microdiets.

Cannibalism was markedly reduced when the larvae were weaned on astaxanthin and phycocyanin based diets. This may be due to better visibility and acceptance of feed particles inducing reduction of prey vulnerability through improved prey escape skills (Baras and Jobling, 2002). Although a varied difference in the cannibalism in the treatment groups was observed, we found no difference in the size heterogeneity of the treatments. This may be because of the reduction in the size heterogeneity due to cannibalism observed in control group and T3 group (Baras and Jobling, 2002).

Immunostimulants offer alternative to drugs in the aquaculture industry, which are used to avoid bacterial disease outbreaks. *V. alginolyticus* is a bacterium of wide distribution, which is known to infect cultured finfish and shellfish (Sharma *et al.*, 2012). We observed reduced mortality after challenge in fish fed phycocyanin and astaxanthin pigmented feeds. The higher resistance to *V. alginolyticus* infection could be attributed to incorporation of astaxanthin and phycocyanin, which are known to have immunostimulant effects when fed through diet (Hayashi *et al.*, 2006; Chuchird *et al.*, 2015).

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