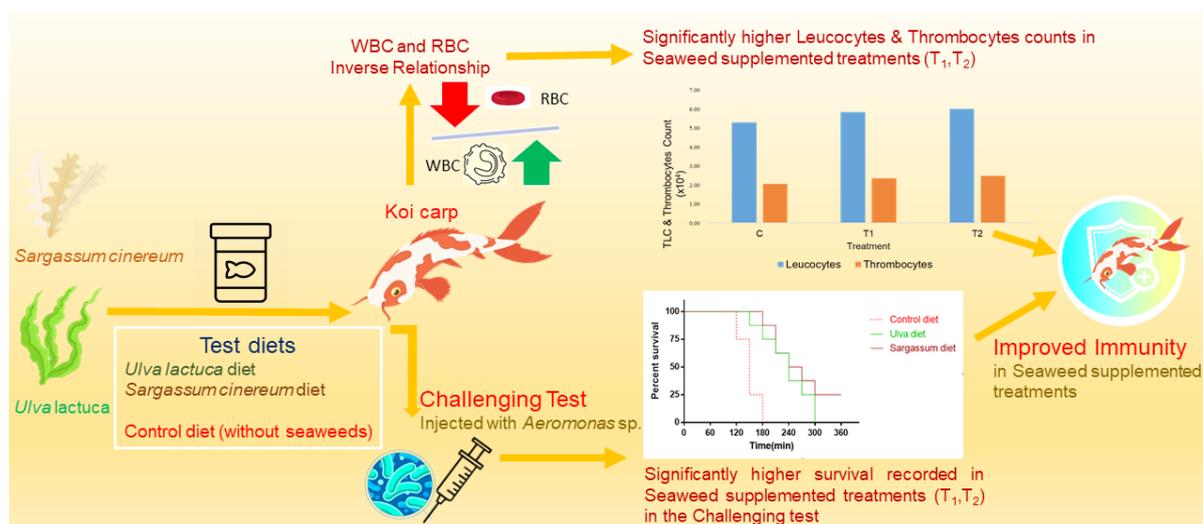


Effects of *Ulva lactuca* and *Sargassum cinereum* supplemented diets on haematological parameters and survival of Koi carp (*Cyprinus carpio* L.) against bacterial pathogen (*Aeromonas* species)

K.G.A. Sudharaka, D.H.N. Munasinghe*, W.A.H.P. Guruge and W.P.R. Chandrarathna



Highlights

- Seaweeds can be used as a cost-effective dietary supplement for Koi carps.
- Diets with *Ulva lactuca* and *Sargassum cinereum* increase survival rates of Koi carps.
- Diets with *Ulva lactuca* and *Sargassum cinereum* affect on blood parameters of Koi carps.
- Seaweeds supplemented diets improved the immunity of Koi carps.

RESEARCH ARTICLE

Effects of *Ulva lactuca* and *Sargassum cinereum* supplemented diets on haematological parameters and survival of Koi carp (*Cyprinus carpio* L.) against bacterial pathogen (*Aeromonas* species)

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Abstract: The objective of the present study was to determine effects of *Ulva lactuca* and *Sargassum cinereum* supplemented diets on some selected blood parameters and survival of fry stage Koi carp (*Cyprinus carpio* Linnaeus, 1758). Fish were fed *Ulva lactuca* diet (T₁), *Sargassum cinereum* diet (T₂) and Control diet (C) for 14 weeks. Haematological parameters were measured at the end of the indoor experiment. Total erythrocyte count (TEC), total leucocyte count (TLC) and thrombocyte count were in the range of 1.44-1.49×10⁶ /mm³, 5.30-6.01×10⁴ /mm³ and 2.05-2.48×10⁴ /mm³ respectively. Thrombocyte count and TLC in T₁ and T₂ were significantly higher ($p < 0.05$) than the control. A challenge-test was conducted to determine their survival against *Aeromonas* species. Two treatments (T₁, T₂) and the control (C) were included into experimental setup in triplicates and each treatment consisted with two groups; saline injected and bacteria injected groups. For each replicate eight healthy Koi carps were introduced. It was revealed that survivorship curves and median survival of seaweed supplemented treatments were significantly higher ($p < 0.05$) from C. The highest survival percentage (25%) in the challenge-test was recorded in T₂ at the censored point after 6 hours.

Keywords: Koi carp; seaweed supplemented diets; haematological parameters; challenge-test; survival.

INTRODUCTION

The determination of hematological reference values is much more difficult in fish than in higher vertebrates due to their poikilothermy and its high spatial, temporal and individual variability (Witeska *et al.*, 2016). Exogenous factors, such as diseases, stress and fishery management always induce major changes in blood composition (Satheeshkumar *et al.*, 2012). Variations in blood parameters depend on the factors such as fish species and its age, sexual maturity, health status as well as aquatic biotope (Fazio *et al.*, 2013; Kandeepan, 2014). Generally, blood contains 1.3 to 7% of the total body weight of fish and it contains active components that help metabolic processes using the gas that exchanges between the organism and the environment.

The occurrence of disease is a result due to complex interactions among the host, pathogen, and the environment (Hedrick, 1998). The most common disease among ornamental fishes is the bacterial infection mainly caused by gram-negative organisms (Lewbart, 2001).

Due to increasing number of diseases, use of antimicrobial agents has been increased dramatically in aquaculture practices (Burrige *et al.*, 2010). Chemotherapeutants, parasiticides, oxidants, biocides, algicides, and herbicides are common types of antimicrobial agents used in aquaculture and finding the right balance between success and failure depends on the accurate diagnosis, dosage rate and the most appropriate method of administration (Rodgers and Furones, 2009). *Aeromonas* sp. (Family: Aeromonadaceae) are gram-negative, straight, non-spore forming (non-porous), generally, cytochrome oxidase-positive, facultative anaerobic and chemoorganotrophic rods. There is now a growing demand for the selection of therapeutic drugs from natural products. Marine organisms, in particular algae, are of immense interest because they possess a wide variety of biological activities such as antibacterial, antifungal, antiviral, antitumor, anti-inflammatory and antioxidant properties (Domestila *et al.*, 2013; Saritha *et al.*, 2013; Parmar *et al.*, 2016; Sathya *et al.*, 2017).

Many algae have bioactive components that inhibit the growth of certain bacterial pathogens (Saritha *et al.*, 2013). Compounds with antifungal and antibacterial activities have been detected in green and brown algae (Lindequist *et al.*, 2001; Newman *et al.*, 2003; Sathya *et al.*, 2017). Due to the overutilization of antibiotics to control the diseases of aquaculture without having any knowledge about the repercussions, microorganisms have developed new strategies to evade the action of antibiotics and consequently, multiple bacterial strains resistant to drugs have evolved (Pérez *et al.*, 2016; Cheesman *et al.*, 2017). Therefore, there is a need to develop more effective and affordable natural antimicrobial agents with better potential and greater efficacy, fewer adverse effects

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than antibiotics, high bioavailability and low toxicity (Thanigaivel *et al.*, 2015). However, the utilization of seaweed as supplementary material in diet preparation in the ornamental fish industry is not popular in Sri Lanka (Coppejans *et al.*, 2009; Tacon and Metian, 2013). The study aims to determine the effects of two types of seaweeds; *Ulva lactuca* and *Sargassum cinereum* supplemented diets on the selected haematological parameters and survival of Koi carp (*Cyprinus carpio* Linnaeus, 1758). Therefore, the present study would form a baseline data as well as reference point for future studies.

MATERIALS AND METHODS

Ethical approval

Ethical clearance for the study was obtained from the Institute of Biology (IOB), Sri Lanka (Permit No: ERC IOBSL 181 11 18).

Preparation of diets

Two test diets; *Ulva lactuca* (T₁), *Sargassum cinereum* (T₂) and the Control [diet without seaweed powder (C)] were formulated according to the Pearson square method (Wagner and Stanton, 2012). Proximate analyses were carried out for ingredients and prepared diets using standard methods (AOAC, 2000 and Ergün *et al.*, 2010). Proportions of ingredients per 1 kg of diet and their percentages used in control diet formulation and the summary of proximate analysis of prepared diets are shown in Table 1 and Table 2 respectively.

Thirty percent of the weight of the control diet was replaced by the *Ulva lactuca* and *Sargassum cinereum* powder to prepare test diets (T₁ and T₂). In order to make 1 kg of T₁ and T₂ test diets, 700 g of control diet was mixed with 300 g of *Ulva lactuca* powder and *Sargassum cinereum* powder respectively. The measured amounts of dietary ingredients were used with a small quantity of dissolved gelatin (binding agent).

Preparation of basic experimental setup for feeding

The experimental set up consisted of two treatments; *Ulva lactuca* supplemented treatment (T₁), *Sargassum cinereum* supplemented treatment (T₂) and the control (C) in triplicates (n = 20 per tank, experimental unit = 150 L fiber tank); Fourteen weeks indoor experiment was carried out using Koi carp fry belonging to the same cohort.

Haematological studies

Three healthy Koi carps (weight = 4.54 ± 0.17 g; length = 5.16 ± 0.07 cm) were randomly selected from each treatment to analyze haematological parameters. The collected blood samples during the haematological studies were immediately subjected to haematological analysis. The blood was drawn from the caudal vein by introducing disposable sterile syringe and 6% EDTA was used as an anticoagulant to prevent the blood cells from lysis and clotting. Different blood parameters viz. total erythrocyte count (TEC), total leucocyte count (TLC) and thrombocyte count were determined using a haemocytometer following Sharma *et al.* (2013).

Experimental setup for challenge-test

Koi carps were given a 2.5 ppm KMnO₄ bath prior to a week of injection of bacteria, to remove external parasites if there were any. Healthy Koi carps (weight = 4.54 ± 0.17 g, length = 5.16 ± 0.07 cm) were kept in KMnO₄ treated quarantine tanks for a period of five days to determine their disease-free health status as described by Citarasu *et al.* (2011). The experiment consisted of two treatments (T₁, T₂) and a control (C) in triplicates and each treatment having two groups namely; 'saline-injected' group (indicate as 's') and 'bacterial broth-injected' group (indicate as 'b'). Each replicate unit was consisted of, 20 L tank (depth = 25 cm; width = 36 cm) and 08 Koi carps. Each treatment carried three replicates representing 'saline-injected' group: (T₁s₁, T₁s₂, T₁s₃)/ (T₂s₁, T₂s₂, T₂s₃)/ (Cs₁, Cs₂, Cs₃) and 'bacterial

Table 1: Proportions of ingredients per 1 kg of diet and their percentages used in control diet formulation

Ingredients	Amount (g) per 1 kg of diet	Percentage (%)
Fish meal	415.72	41.572
Soybean	415.72	41.572
Coconut meal	77.78	7.778
Wheat flour	77.78	7.778
Vitamin and Mineral mixture	13.00	1.300

Table 2: Types of diets and proximate compositions of experimental diets

Proximate Analysis	Diet		
	C	T ₁	T ₂
Dry matter (%)	90.00 ± 0.10 ^b	92.72 ± 0.23 ^a	92.05 ± 0.35 ^a
Ash (%)	23.06 ± 0.14 ^c	38.17 ± 0.26 ^a	26.73 ± 0.19 ^b
Crude Protein (%)	36.26 ± 0.57 ^a	24.14 ± 0.27 ^b	21.33 ± 0.01 ^c
Lipid (%)	6.95 ± 0.02 ^a	4.62 ± 0.14 ^c	5.59 ± 0.21 ^b

Values are means ± SEM (n = 3). Different letters in same line indicate significant differences among treatment groups ($p < 0.05$).

broth-injected' group: (T₁b₁, T₁b₂, T₁b₃)/(T₂b₁, T₂b₂, T₂b₃)/(Cb₁, Cb₂, Cb₃).

Sub-culturing of *Aeromonas* sp.

Prior to two days of injecting *Aeromonas* sp. to Koi carps, prepared TSA plates were used to grow out subculture colonies using a pure culture of *Aeromonas* sp. Tryptic Soy Agar (commercial brand: HIMEDIA® M1968-500G) media plates were prepared according to the procedure given in the HIMEDIA® Technical Data Sheet (Himedia Laboratory, 2020). The pathogen was inoculated using a sterile loop following the streak plate method (Citarasu et al., 2011). Then the TSA plate was inserted in a sealed polythene bag and kept inverted in an incubator for 24 hours at 37 °C following Citarasu et al. (2011).

Preparation of nutrient broth and dilution series

In the preparation of nutrient broth solution, 0.65 g of nutrient broth powder was measured (electronic balance: Mettler PE 3600, weight ± 0.01) followed by dissolving the measured amount in 50 mL of distilled water in a sterile 100 mL conical flask using a magnetic stirrer under sterile laboratory conditions. Its mouth was covered with a cotton plug and tin foil respectively, and autoclaved at 15 lbs pressure (121 °C) for 15 minutes and kept near to a spirit lamp flame. The sub-cultured plate was taken out of the incubator and a loop full of pure colony was taken from the sub-cultured plate and it was dissolved well in the prepared broth solution. Dilution series were prepared on the same day of nutrient broth preparation. In the preparation of dilution series, 1.0 mL of original nutrient broth solution was taken using a micropipette and placed into the 9.0 ± 0.1 mL of distilled water in a 15 mL graduated glass test tube to make 10.0 mL of diluted solution. The resulting diluted solution contained 1.0 mL of 10-fold diluted original inoculum in nutrient broth. Likewise, a 10-fold dilution series was made up to 10⁻⁶ dilution. Then 100 ± 1 µL was taken from each concentration and placed the solution drop on separate TSA plates using a micropipette and followed by spreading with a glass spreader following Sanders (2012). All TSA plates were thoroughly examined in order to make sure that the solution is uniformly spread and incubated in sealed airbags for 24 hours at 37 °C following the method described by Citarasu et al. (2011). The conical flask with the remaining portion of nutrient broth solution was covered with a cotton plug and sealed with tin foil. Then it was kept in a laboratory shaker with an adjusted value of 80 rpm at 36 °C for 18 hours. Bacterial enumeration was carried out using spread plate method (Hedges et al., 1978).

Challenge-test

After feeding fish in two treatments (T₁, T₂) and control (C) with prepared diets for 14 weeks, fish in treatment tanks were inoculated with the *Aeromonas* sp. (bacterial pathogen) and the challenge-test was conducted (Raghuathan et al., 2014). Each individual of Koi carps in Cb, Ub and Sb were injected with 0.2 mL of bacterial broth while Koi carps in Cs, Us and Ss were injected with normal saline. Injections were given intra-peritoneally using 1 ±

0.1 mL Terumo™ syringes. The behaviour of Koi carps was observed in bacteria injected and saline-injected tanks after the injection and abnormal behaviours were recorded.

Survival of Koi carps

Survival data (number of survivors) of two treatments (T₁, T₂) and the control (C) were recorded in half an hour time interval for six hours. Died specimens were dissected and checked for pathogenic bacteria using re-isolation techniques.

Observation of behavior of Koi carps

Immediately after administering the broth containing *Aeromonas* sp. and saline, the behaviour of Koi carps in all treated tanks was observed for six hours continuously. Abnormal behaviours (air gulping, lethargy and erratic swimming etc.) were recorded.

Re-isolation of the bacterial pathogen from Koi carps

Re-isolation of *Aeromonas* sp. from fish organs was carried out to confirm that fish were infected and deceased due to *Aeromonas* sp. Autoclaved vials (at 15 lbs pressure, 121 °C for 15 minutes) were used to collect dead fish specimens. Three dead fish were selected from two treatments and control for examinations. External manifestations of bacterial infection were checked prior to dissection of dead fish specimens. Three TSA plates were used and each TSA plate was divided into six regions externally using a marker. Subscripted letters 1, 2 and 3 were used to symbolize fish specimen 1, fish specimen 2, fish specimen 3 and uppercase letters 'L' and 'K' were used to denote liver and kidney respectively. Six regions of the TSA plate for T₂ treatment were marked as Sb₁L, Sb₁K, Sb₂L, Sb₂K, Sb₃L, Sb₃K and likewise six regions of the TSA plate for T₁ treatment were marked as Ub₁L, Ub₁K, Ub₂L, Ub₂K, Ub₃L, Ub₃K. Similarly, the six regions of the TSA plate for control were also marked as Cb₁L, Cb₁K, Cb₂L, Cb₂K, Cb₃L, Cb₃K. Fish were dissected and kidney and liver were touched with a sterile inoculating loop and streaked on designated regions of previously prepared TSA plates. Then the TSA plates were inserted in a sealed polythene bag and kept inverted in an incubator for 24 hours at 37 °C.

Identification and confirmation of the re-isolated bacterial pathogen

Morphological characteristics of isolates, Gram staining procedure as described by Nahar et al. (2016) and Nigrosin staining method were followed in identifying re-isolated bacteria. Photographs were taken using the OLYMPUS BX43 upright microscope (Japan, model U-CBS). Photographs were compared with published literature (Abbott et al., 2003; Nahar et al., 2016; Hardy diagnostics catalogue, 2019) to ensure that the re-isolated bacterial pathogen was identical to the injected bacterial type.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze haematological parameters. All calculations were carried out using the statistical software Minitab 17.0 and Microsoft Excel 2016 for windows 8.1. The significance

level of $p < 0.05$ at 95% confidence limit was used to check the significant difference between treatments and the control for haematological parameters by one-way ANOVA and Tukey multiple range test.

The survival percentage of two treatments and control were plotted against time to determine the percentage of survival. Significant differences among Kaplan-Meier survivorship curves for two treatment tanks and control were statistically analyzed ($p < 0.05$) using Log-rank (Mantel-Cox) test for pairwise comparisons with the help of GraphPad Prism 6.01 software (Yilmaz *et al.*, 2013).

RESULTS

Haematological parameters

Total erythrocyte count (TEC) varied from 1.44×10^6 - 1.49×10^6 /mm³ while the total leucocyte count (TLC) ranged from 5.30×10^4 - 6.01×10^4 /mm³. The thrombocyte count was in the range of 2.05×10^4 - 2.48×10^4 /mm³ (Table 3). The lowest TEC obtained in T₂ while the highest TEC recorded in the control group. TEC of T₁ and T₂ was not significantly different ($p > 0.05$). However, the TEC of control group was significantly different ($p < 0.05$) from the two treatments. There was an inverse relationship between the number of leucocytes and erythrocytes in Koi carps in C, T₁ and T₂.

The lowest thrombocyte (Platelet) count (2.05×10^4 /mm³) and the highest thrombocyte count (2.48×10^4 /mm³) were recorded in the control group and T₂, respectively. Thrombocyte count and TLC in seaweed supplemented treatments (T₁ and T₂) were significantly higher ($p < 0.05$) than those in control. Furthermore, all blood parameters (TEC, TLC and thrombocyte count) in control group were significantly different ($p < 0.05$) from T₂.

Effect on survival of Koi carps in T₁, T₂ and C in the challenge- test

Mortalities were only recorded in *Aeromonas* sp. injected groups. No fish were dead in saline-injected groups. The Kaplan-Meier survivorship curves for Koi carps in challenged groups (T₁, T₂ and C) are shown in Figure 1.

The lowest survival time (180 min) was recorded in the control while the second highest survival time (300 min) survival time recorded in T₁. The highest survival percentage (25%) was recorded in T₂ at the censored point after 06 hours while the control and T₁ reached 100% mortality within 180 min and 300 min respectively (Figure 1).

The Log-Rank (Mantel-Cox) test for pairwise comparison of survival curves between C and T₂ were significantly different ($p < 0.05$) with a median survival time of 150 min and 255 min respectively. The survival time of Koi carps in the control group was lower than the survival time of *Sargassum* diet fed (T₂) koi carps.

The Log-Rank (Mantel-Cox) test for pairwise comparison of survival curves between C and T₁ were significantly different ($p < 0.05$) with a median survival time of 150 min and 240 min respectively. The survival time of Koi carps in the control was lower than the survival time of *Ulva* fed (T₁) Koi carps.

The Log-Rank (Mantel-Cox) test for pairwise comparison of survival curves between T₁ and T₂ treatments were not significantly different ($p > 0.05$). The median survival times for T₁ and T₂ were 240 min and 255 min respectively.

Figure 1: Kaplan-Meier survivorship curves plotted for Koi carps fed with Control diet (C), *Ulva* diet (T₁) and *Sargassum* diet (T₂) in the challenge-test

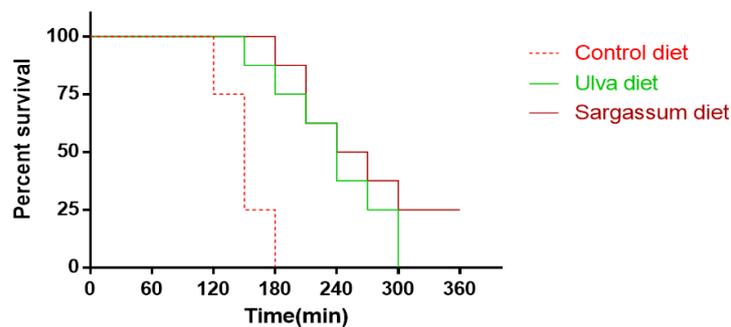


Table 3: Selected blood parameters of Koi carps in two treatments (T₁, T₂) and control (C)

Blood Parameter	Group		
	C	T ₁	T ₂
Total Erythrocyte Count (TEC) ($\times 10^6$ /mm ³)	1.49 ± 0.01 ^a	1.46 ± 0.01 ^{ab}	1.44 ± 0.01 ^b
Total Leucocyte Count (TLC) ($\times 10^4$ /mm ³)	5.30 ± 0.03 ^c	5.85 ± 0.02 ^b	6.01 ± 0.02 ^a
Thrombocyte Count ($\times 10^4$ /mm ³)	2.05 ± 0.03 ^c	2.35 ± 0.02 ^b	2.48 ± 0.01 ^a

Values are means ± SEM (n = 9). Different letters in same row indicate significant differences within groups ($p < 0.05$).

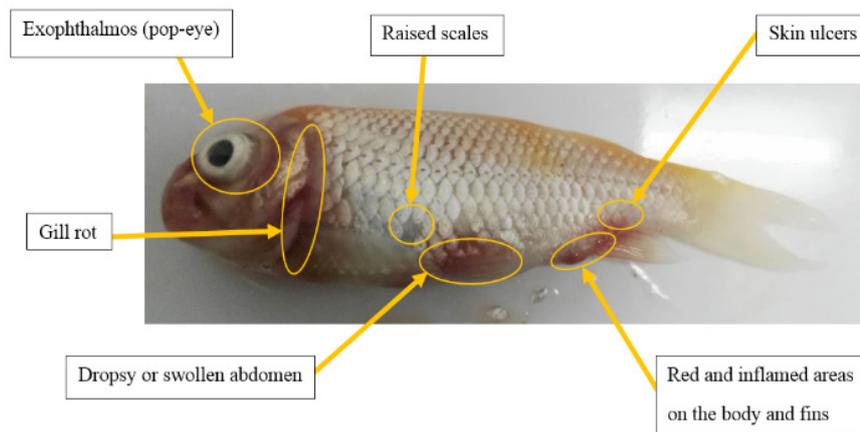


Figure 2: External symptoms of a dead Koi carp after 06 hours of injection with *Aeromonas* sp.
Note: The areas with external symptoms are circled in yellow colour to indicate clearly (*size is not proportionate*).

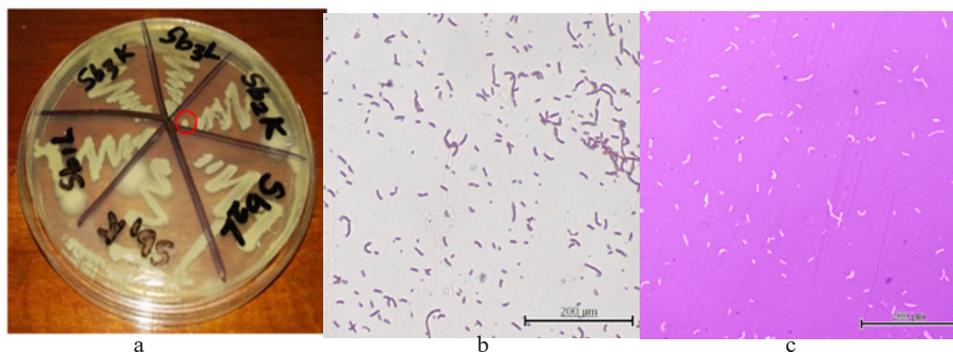


Figure 3: Re-isolation and identification of *Aeromonas* sp. [a] Re-isolated bacterial pathogen from liver and kidneys of a bacteria injected fish in *Sargassum* treatment [b] Gram-stained re-isolated bacterial pathogen; [c] Nigrosin stained re-isolated bacterial pathogen.
Note: An isolated colony used to examine the bacterial pathogen is indicated by a red colour circle in Figure 3[a].

Behavioral observations of Koi carps injected with *Aeromonas* sp.

Abnormal behavioural observations such as air gulping behaviour, sluggishness and lethargy and erratic swimming with alternate floating and sinking followed by temporary rejuvenation were observed. This pattern was repeated until the death occurred eventually.

Specimen collection for re-isolation

External symptoms such as gill rot, inflamed red patches on the body and fins, raised or elevated scales, skin ulcers, exophthalmos (pop-eye), dropsy abdomen (swollen belly) were observed in collected specimens of dead Koi carps after injection of *Aeromonas* sp. (Figure 2).

Re-isolation and identification of *Aeromonas* sp.

The re-isolated bacterial pathogen from the liver and kidneys of dead fish specimens were successfully grown up (Figure 3a). The bacterial pathogen re-isolated from T₁, T₂ and C were Gram-negative and exhibited morphological characters such as straight cells, rod-shaped with rounded ends to coccoid (Figure 3b). They appeared alone, in pairs, or in short chains. The shape of the re-isolated bacteria was observed as rod-shaped by Nigrosin staining, and it was confirmed that the re-isolated bacterial pathogen was as same as the injected bacterial pathogen (*Aeromonas* sp.) (Figure 3c).

DISCUSSION

The objective of the study was to determine the effect of two types of seaweeds; *Ulva lactuca* and *Sargassum cinereum*, supplemented diets on the survival of Koi carps (*Cyprinus carpio* Linnaeus, 1758). According to the previous studies *Aeromonas* sp. are the most commonly found pathogenic bacterial genus that infest on ornamental fish in Sri Lanka (Hettiarachchi and Cheong, 1994; Pathiratne *et al.*, 1994). Based on this information *Aeromonas* sp. was selected as the suitable bacterial candidate for the current experiment. *Aeromonas* sp. is one of the best studied species which has the potential to cause sub-acute to chronic disease in Koi carp and other cyprinids (Behrmann-Godel, 2015). *Aeromonas* sp. is reported to be the causative agent of haemorrhagic septicaemia, ulcer disease (red-sore disease) and motile aeromonad septicemia (Adanir and Turutoglu, 2007; Bhuvanawari *et al.*, 2018).

In a similar study carried out by Witeska *et al.* (2016) using 5-8 months old clinically healthy Common carp juveniles suggested blood cell count reference values for erythrocytes, leucocytes and thrombocytes were in the range of $1.35-1.51 \times 10^6 / \text{mm}^3$, $5.13-6.08 \times 10^4 / \text{mm}^3$ and $1.44-2.54 \times 10^4 / \text{mm}^3$ respectively. The results of the present study are in accordance with the findings of Witeska *et al.* (2016).

The results showed that the number of leucocytes

appears to have a wide range of variation from $5.30 \times 10^4 / \text{mm}^3$ to $6.01 \times 10^4 / \text{mm}^3$. The lowest TLC value ($5.30 \times 10^4 / \text{mm}^3$) could be seen in the control group and the highest value obtained in T_2 . Total leucocyte counts in seaweed supplemented treatments (T_1 and T_2) were significantly higher ($p < 0.05$) than the control group. Leucocytes (White Blood Cells) are mainly responsible for immune responses of animals. Species with greater amounts of Leucocyte are more resistant for infections than those with lower levels (Kandeepan, 2014). Therefore, among the studied seaweed supplemented treatments and the control, Koi carps in seaweed supplemented treatments would be able to fight infection more effectively than the control group.

In a similar study, Eyiwunmi *et al.* (2018) showed that there was an increase in leucocytes of the experimental *Clarias gariepinus* (African sharp-tooth catfish) fed with varying *Moringa oleifera* leaf meal and this increment in leucocytes in the circulatory system helped to increase the survival rate of the experimental fish. Similarly, according to the results of the present study it can be suggested that increase in leucocytes in Koi carps fed with seaweed supplemented diets (T_1 and T_2) had a potential impact on survival of juvenile Koi carps in the challenge-test. High differences in leucocytes may have resulted from different leukocyte counting procedures other than haemocytometer method which was used in the present study to count all three blood cell types; erythrocytes, leucocytes and thrombocytes (Witeska *et al.*, 2016).

All the TEC values were within the range of standard values as suggested by Witeska *et al.* (2016). An inverse relationship between the number of leucocytes and erythrocytes in Koi carps fed with seaweed supplemented treatments (T_1 and T_2) and in control (C) could be observed in present study. The same inverse relationship between leucocytes and erythrocytes has been found by Satheeshkumar *et al.* (2012) and Kandeepan (2014). It is well documented that a higher number of erythrocytes may reduce the need for a large number of leucocytes (Zhou *et al.*, 2009; Satheeshkumar *et al.*, 2012).

Fish thrombocytes have been found to have phagocytic capability and to engage in defensive mechanisms (Stosik *et al.*, 2001; Kandeepan, 2014) and it has been found that fish thrombocytes are blood phagocytes which form a protective barrier (Kandeepan, 2014). Therefore, it can be suggested that extra level of protection against pathogens can be obtained with higher thrombocyte counts and, given that it can be shown that seaweed supplemented diet fed fish may have better survival probability than the control diet fed fish in a challenge-test.

Witeska *et al.* (2016) concluded that there may be differences in haematological parameters of fish reared in laboratory conditions and directly taken from the natural environment. The transfer of fish from the natural environment to the laboratory inevitably involves a certain degree of stress due to handling and confinement even if an appropriate acclimatization takes place. There are no single species that can be used as a representative model for bony fishes, as fish show numerous adaptations to various habitats during their evolutionary history. Therefore,

further haematological assays and studies must be carried out for the better understanding of fish immunity.

Most external manifestations and symptoms observed during the present study were consistent with past studies. According to the past studies (Adanir and Turutoglu, 2007; Oliver, 2010; Behrmann-Godel, 2015; Bhuvaneshwari *et al.*, 2018) *Aeromonas* bacterial infections showed symptoms such as enlarged eyes (exophthalmos), fluid buildup in the abdomen (ascitis), renal dropsy (kidney damage), ragged fins, reddening of the body, and hemorrhagic patches in the gills, tail, fins, body wall, and internal organs, skin and gill ulcers, epidermal lesions, fin or tail rot, bleeding at the base of fins, epithelial necrosis and loss of appetite (Adanir and Turutoglu, 2007; PetMD Editorial, 2008; Behrmann-Godel, 2015; Bhuvaneshwari *et al.*, 2018). Adanir and Turutoglu (2007) observed the separation of the basement membrane from the overlying epidermis of the skin with loss of the specialized cells such as mucus and club cells giving a ballooning impression. Further, it has been found that infections occur mainly in the spring and summer when temperature rises. *Aeromonas* sp. are thought to be dormant at temperatures below 4 °C. Temperature stress, on the other hand, causes activity and infection rates to increase exponentially (Oliver, 2010; Behrmann-Godel, 2015).

In the Bacterial enumeration, the 10^{-6} TSA plate which contained 52 colonies was chosen to calculate Colony Forming Units per milliliter (CFU/ mL). With the dilution factor of 10^6 and the 100 μL volume spread on the culture plate 5.2×10^8 CFU/ mL was obtained. As 1 mL of broth contained 5.2×10^8 CFU, injected volume (0.2 mL) of broth contained 1.04×10^8 CFU. Therefore, the lethal concentration may lead to the mortality of fish within such a short time period. But, Koi carps in the *Sargassum* treatment managed to survive the longest period of all treatments. This may be due to the higher antioxidant and immuno-stimulant properties of *Sargassum* sp. used to formulate that test diet. Gora *et al.* (2018) carried out a similar experiment to show the effect of dietary *Sargassum wightii* extract on immunity, disease resistance and antimicrobial peptide gene expression in *Labeo rohita* and the bacterial challenge-test using *Aeromonas hydrophila* showed higher survival rates and better non-specific immune response in the *Sargassum wightii* extract fed groups. The results obtained by Gora *et al.* (2018) are in accordance with the present study. In a previous study, Hwang *et al.* (2010) described the antioxidant and immune-stimulating activities of hot-water extract from brown seaweed, *Sargassum hemiphyllum*. Huang *et al.* (2006) showed that the immune-stimulatory effects of some polysaccharide extracts of *Sargassum fusiforme* for control of shrimp diseases (vibriosis). Yangthog *et al.* (2016) showed that an aqueous extract from *Sargassum* sp. can be used to enhance the immune response and resistance against *Streptococcus iniae* in the Asian sea bass (*Lates calcarifer* Bloch, 1790). These findings suggest that *Sargassum* sp. supplementations or extracts have a potential impact on immune responses which support the findings of the current study. There was a similar study that described on the non-specific immune potentiating activity of fucoidan from a tropical brown algae (Phaeophyceae), *Sargassum cristaefolium* in *Oreochromis*

niloticus (Isnansetyo et al., 2014). Therefore, it is evident that brown seaweeds are rich in chemical compounds that enhance the immune system. In the present study, brown seaweed *Sargassum cinereum* has significantly increased the survival of Koi carps in the challenge-test. This may be due to the enhancement of immunity by potential immunostimulants in *Sargassum cinereum*.

Finally, it could be suggested that if this experiment could be conducted for a one-year period, more informative results on patterns of fluctuation of growth indices could be collected. Also, more studies must be conducted to determine the effect of seaweed supplementation in different percentages to the basal diet on the growth performance of fish.

CONCLUSION

An inverse relationship was observed between the number of leucocytes and erythrocytes in Koi carps fed with seaweed supplemented treatments (T₁, T₂) and the control (C). Therefore, it can be suggested that the use of seaweed supplemented diets has a potential impact on survival time through the development of immunity in Koi carps under laboratory conditions and given that it can be suggested that the use of seaweed supplemented diets support to increase longevity through the development of immunity in Koi carps. The results showed the potential use of *Ulva lactuca* and *Sargassum cinereum* as a dietary supplement in aquaculture programmes. Further studies are suggested to incorporate other substances (such as digestive enzymes) when formulating seaweed supplemented diets to increase the effectiveness of the diets.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interests.

REFERENCES

- Abbott, S.L., Cheung, W.K. and Janda, J.M. (2003). The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *Journal of Clinical Microbiology* **41**(6): 2348-2357. DOI: <https://doi.org/10.1128/JCM.41.6.2348-2357.2003>.
- Adanir, D.O.R. and Turutoglu, H. (2007). Isolation and antibiotic susceptibility of *Aeromonas hydrophila* in a carp. *Bulletin of the Veterinary Institute in Pulawy* **51**: 361-364.
- AOAC (2000). *Association of Official Analytical Chemists (AOAC)*, 17th (ed). Washington, D.C.
- Behrmann-Godel, J. (2015). Disease Agents and Parasites of Carp. In: Pietsch C.; Hirsch P. (Eds.), *Biology and Ecology of Carp*, Boca Raton, CRC Press, Taylor & Francis Group. Pp.174-176.
- Bhuvaneshwari R., Manickam N. and Santhanam P.

- (2018). Recovery of *Cyprinus Carpio* (Ornamental Koi Carp) experimentally infected with *Aeromonas hydrophila* through Phytotherapy. *Journal of Aquatic Research and Marine Sciences*. 55-68. DOI: <https://doi.org/10.29199/2639-4618/ARMS.101014>.
- Burrige, L., Weis, J.S., Cabello, F., Pizarro, J. and Bostick, K. (2010). Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. *Aquaculture* **306**(1-4): 7-23. DOI: <https://doi.org/10.1016/j.aquaculture.2010.05.020>.
- Cheesman, M.J., Ilanko, A., Blonk, B. and Cock, I.E. (2017). Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution?. *Pharmacognosy Reviews* **11**(22): 57-72. DOI: https://doi.org/10.4103/phrev.phrev_21_17.
- Citarasu, T., Dhas, A., Velmurugan, S., Viji, T.V., Kumaran, T., Babu, M.M. and Selvaraj, T. (2011). Isolation of *Aeromonas hydrophila* from infected ornamental fish hatchery during massive disease outbreak. *International Journal of Current Research* **2**(1): 37-41.
- Coppejans, E., Leliaert, F., Dargent, O., Gunasekara, R. and De Clerck, O. (2009). *Sri Lankan Seaweeds: Methodologies and field guide to the dominant species. ABC Taxa (Vol. 6)*. Brussel: Belgian National Focal Point to the Global Taxonomy Initiative, 265.
- Domettilla, C., Brintha, T.S.S., Sukumaran, S. and Jeeva, S. (2013). Diversity and distribution of seaweeds in the Mutton coastal waters, south-west coast of India. *Biodiversity Journal* **4**(1): 105-110.
- Ergün, S., Güroy, D., Tekeşoğlu, H., Güroy, B., Çelik, İ., Tekinay, A.A. and Bulut, M. (2010). Optimum dietary protein level for Blue Streak Hap (*Labidochromis caeruleus*). *Turkish Journal of Fisheries and Aquatic Sciences* **10**(1): 27-31. DOI: <https://doi.org/10.4194/trjfas.2010.0104>.
- Eyiwunmi, F.A., Augustine, O. and Ovie, W.K. (2018). The Hematological parameters of Catfish (*Clarias gariepinus*) fed Fish Feeds with replaced Premix using Moringa Leaf Meal (MLM). *Madridge Journal of Aquaculture Research and Development* **2**(1): 35-39. DOI: <https://doi.org/10.18689/mjard-1000107>.
- Fazio, F., Marafioti, S., Arfuso, F., Piccione, G. and Faggio, C. (2013). Comparative study of the biochemical and haematological parameters of four wild Tyrrhenian fish species. *Veterinari Medicina* **58**(11): 576-581. DOI: <https://doi.org/10.17221/7139-VETMED>.
- Gora, A.H., Sahu, N.P., Sahoo, S., Rehman, S., Dar, S.A., Ahmad, I. and Agarwal, D. (2018). Effect of dietary *Sargassum wightii* and its fucoidan-rich extract on growth, immunity, disease resistance and antimicrobial peptide gene expression in *Labeo rohita*. *International Aquatic Research* **10**(2): 115-131. DOI: <https://doi.org/10.1007/s40071-018-0193-6>.
- Hardy diagnostics catalogue (2019). *Description of the genus Aeromonas*, Hardy diagnostics, 1430 West McCoy Lane, Santa Maria, CA 93455, USA. Available from: http://catalog.hardydiagnostics.com/cp_prod/Content/hugo/Aeromonas.htm (Accessed 6 April 2019).
- Hedges, A.J., Shannon, R. and Hobbs, R.P. (1978).

- Comparison of the precision obtained in counting viable bacteria by the spiral plate maker, the droplette and the Miles & Misra methods. *Journal of Applied Bacteriology* **45**(1): 57-65. DOI: <https://doi.org/10.1111/j.1365-2672.1978.tb04198.x>.
- Hedrick, R.P. (1998). Relationships of the host, pathogen, and environment: implications for diseases of cultured and wild fish populations. *Journal of Aquatic Animal Health* **10**(2): 107-111. DOI: [https://doi.org/10.1577/1548-8667\(1998\)010<0107:ROTHPA>2.0.CO;2](https://doi.org/10.1577/1548-8667(1998)010<0107:ROTHPA>2.0.CO;2).
- Hettiarachchi, D.C. and Cheong, C.H. (1994). Some characteristics of *Aeromonas hydrophila* and *Vibrio* species isolated from bacterial disease out-breaks in ornamental fish culture in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka* **22**(3): 261-269. DOI: <http://doi.org/10.4038/jnsfsr.v22i3.8128>.
- Himedia Laboratory (2020). *Himedia Technical Data Sheet*, HiMedia Laboratories LLC, 107 W Dorothys Way, Lincoln University, West Chester, Pennsylvania, USA. Available from: <http://himedialabs.com/TD/M1968.pdf/> (Accessed 15 July 2020).
- Huang, X., Zhou, H. and Zhang, H. (2006). The effect of *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the shrimp, *Fenneropenaeus chinensis*. *Fish and Shellfish Immunology* **20**(5): 750-757. DOI: <https://doi.org/10.1016/j.fsi.2005.09.008>.
- Hwang, P.A., Wu, C.H., Gau, S.Y., Chien, S.Y. and Hwang, D.F. (2010). Antioxidant and immune-stimulating activities of hot-water extract from seaweed *Sargassum hemiphyllum*. *Journal of Marine Science and Technology* **18**(1): 41-46.
- Isnansetyo, A., Irfani, H.M., Wulansari, T.A. and Kasanah, N. (2014). Oral Administration of Alginate from A Tropical Brown Seaweed, *Sargassum* sp. to Enhance Non-Specific Defense in Walking Catfish (*Clarias* sp.). *Aquacultura Indonesiana* **15**(1): 14-20. DOI: <http://doi.org/10.21534/ai.v15i1.29>.
- Kandeepan, C. (2014). Haematological and biochemical parameters on few fresh water South Indian Teleosts. *International Journal of Current Microbiology and Applied Sciences* **3**(9): 1015-1022.
- Lewbart, G.A. (2001). Bacteria and ornamental fish. *Seminars in Avian and Exotic Pet Medicine* **10**(1): 48-56. DOI: <https://doi.org/10.1053/saep.2001.19543>.
- Lindequist, U., Sender, U., Bandow, J., Jülich, W.D., Kusnick, C., Schweder, T. and Hecker, M. (2001). Antibacterial substances from marine fungi and their investigation by proteome-based methods. In *Proceedings of 8th International Marine and Freshwater Mycology Symposium*, Hurghada, Egypt, p457.
- Nahar, S., Rahman, M.M., Ahmed, G.U. and Faruk, M.R. (2016). Isolation, identification, and characterization of *Aeromonas hydrophila* from juvenile farmed pangasius (*Pangasianodon hypophthalmus*). *International Journal of Fisheries and Aquatic Studies* **4**(4): 52-60.
- Newman, D.J., Cragg, G.M. and Snader, K.M. (2003). Natural Products as Sources of New Drugs over the Period 1981– 2002. *Journal of Natural Products* **66**(7): 1022-1037. DOI: <https://doi.org/10.1021/np0300961>.
- Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews* **34**(4): 415-425. DOI: <https://doi.org/10.1111/j.1574-6976.2009.00200.x>.
- Parmar, P.V., Solanki, J.B., Nanjiyani, R.P., Kamaliya, N.L., Chavda, V.M., Parmar, H.V. and Bajaniya, V.C. (2016). Antibacterial activity of seaweeds from vera weeds from veraval coast. *The Bioscan* **11**(4): 2133-2135.
- Pathiratne, A., Widanapathirana, G.S. and Chandrakanthi, W.H.S. (1994). Association of *Aeromonas hydrophila* with epizootic ulcerative syndrome (EUS) of freshwater fish in Sri Lanka. *Journal of Applied Ichthyology* **10**(2-3): 204-208. DOI: <https://doi.org/10.1111/j.1439-0426.1994.tb00160.x>.
- Pérez, M., Falqué, E. and Domínguez, H. (2016). Antimicrobial Action of Compounds from Marine Seaweed. *Marine Drugs* **14**(3): 52. DOI: <https://doi.org/10.3390/md14030052>.
- PetMD Editorial (2008). *Bacterial Infections (Aeromonas) in Fish*, Plymouth Meeting, Pennsylvania, USA, Available from: https://www.petmd.com/fish/conditions/skin/c_fi_aeromonas/ (Accessed 23 June 2019).
- Raghunathan, G., Dhayanithi, N.B., Kumar, T.T.A. and Kumaresan, S. (2014). Evaluation of antibacterial activity and immunostimulant of red seaweed *Chondrococcus hornemanni* (Kuetzing, 1847) against marine ornamental fish pathogens. *Journal of Coastal Life Medicine* **2**(1): 64-69. DOI: <https://doi.org/10.12980/jclm.2.2014j46>.
- Rodgers, C.J. and Furones, M.D. (2009). Antimicrobial agents in aquaculture: Practice, needs and issues. *Options Méditerranéennes* **86**: 41-59.
- Sanders, E.R. (2012). Aseptic laboratory techniques: plating methods. *Journal of Visualized Experiments* **63**: 3064. DOI: <https://doi.org/10.3791/3064>.
- Saritha, K., Mani, A.E., Priyalaxmi, M. and Patterson, J. (2013). Antibacterial activity and biochemical constituents of seaweed *Ulva lactuca*. *Global Journal of Pharmacology* **7**(3): 276-282. DOI: <https://doi.org/10.5829/idosi.gjp.2013.7.3.75156>.
- Satheeshkumar, P., Ananthan, G., Kumar, D.S. and Jagadeesan, L. (2012). Haematology and biochemical parameters of different feeding behaviour of teleost fishes from Vellar estuary, India. *Comparative Clinical Pathology* **21**(6): 1187-1191. DOI: <https://doi.org/10.1007/s00580-011-1259-7>.
- Sathya, R., Kanaga, N., Sankar, P. and Jeeva, S. (2017). Antioxidant properties of phlorotannins from brown seaweed *Cystoseira trinodis* (Forsskål) C. Agardh. *Arabian Journal of Chemistry* **10**: 2608-2614. DOI: <https://doi.org/10.1016/j.arabjc.2013.09.039>.
- Sharma, P., Sihag, R.C. and Gahlawat, S.K. (2013). Effect of probiotic on haematological parameters of diseased fish (*Cirrihinus mrigal*). *Journal of Fisheries Sciences.com* **7**(4): 323. DOI: <https://doi.org/10.3153/jfsc.com.2013036>.
- Stosik, M., Deptula, W. and Travnicek, M. (2001). Studies on the number and ingesting ability of thrombocytes in sick carps (*Cyprinus carpio* L.). *Veterinárni Medicina*

- 46(1): 12-16. DOI: <https://doi.org/10.17221/7845-VETMED>.
- Tacon, A.G.J. and Metian, M. (2013). Fish matters: importance of aquatic foods in human nutrition and global food supply. *Reviews in Fisheries Science* **21**(1): 22-38. DOI: <https://doi.org/10.1080/10641262.2012.753405>.
- Thanigaivel, S., Hindu, S.V., Vijayakumar, S., Mukherjee, A., Chandrasekaran, N. and Thomas, J. (2015). Differential solvent extraction of two seaweeds and their efficacy in controlling *Aeromonas salmonicida* infection in *Oreochromis mossambicus*: a novel therapeutic approach. *Aquaculture* **443**: 56-64. DOI: <https://doi.org/10.1016/j.aquaculture.2015.03.010>.
- Wagner, J. and Stanton, T.L., (2012). *Formulating rations with the Pearson square*, Doctoral dissertation, Colorado State University Library, Colorado State University, Cooperative Extension Program, Fact Sheet No 1.618. Available from: https://mountainscholar.org/bitstream/handle/10217/182546/AEXT_ucsu2062216182012.pdf?sequence=1/ (Accessed 23 June 2019).
- Witeska, M., Lugowska, K. and Kondera, E. (2016). Reference values of hematological parameters for juvenile *Cyprinus carpio*. *Bulletin of the European Association of Fish Pathologists* **36**(4): 169.
- Yangthong, M., Hutadilok-Towatana, N., Thawonsuwan, J. and Phromkunthong, W. (2016). An aqueous extract from *Sargassum* sp. enhances the immune response and resistance against *Streptococcus iniae* in the Asian sea bass (*Lates calcarifer* Bloch). *Journal of Applied Phycology* **28**(6): 3587-3598. DOI: <https://doi.org/10.1007/s10811-016-0859-7>.
- Yılmaz, S., Ergün, S. and Soyaş, N. (2013). Dietary supplementation of cumin (*Cuminum cyminum*) preventing streptococcal disease during first-feeding of Mozambique tilapia (*Oreochromis mossambicus*). *Journal of Bioscience and Biotechnology* **2**(2): 117-124.
- Zhou, X., Li, M., Abbas, K. and Wang, W. (2009). Comparison of haematology and serum biochemistry of cultured and wild Dojo loach *Misgurnus anguillicaudatus*. *Fish Physiology and Biochemistry* **35**(3): 435-441. DOI: <https://doi.org/10.1007/s10695-008-9268-4>.
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