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FULL LENGTH ARTICLE

Effects of short term feeding of some marine microalgae on the microbial profile associated with *Dicentrarchus labrax* post larvae

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Abstract This study investigates the microbial profile and antimicrobial activity of four marine microalgae species, *Tetraselmis chuii*, *Nannochloropsis salina*, *Isochrysis galbana* and *Chlorella salina* used in aquaculture of *Dicentrarchus labrax* in the post larval stage to estimate which was the best algal species that could be used as a green water technique and achieving the maximum rate of growth and survival of *D. labrax* post larvae. The results represented a significant increase in the length and width of *D. labrax* at $p < 0.05$ recorded in the case of enrichment with *I. galbana* followed by *N. salina*, and the most weight was recorded in the case of *N. salina* as compared with the control. Significant increase in percentage of survival of *D. labrax* was recorded in the case of *C. salina* and *T. chuii* (70% and 60.1%, respectively) as compared with the control (22%). The antibacterial activity (AU) of the different microalgal ethanolic extracts against fish indicator pathogens was determined. The results indicated that the ethanolic extracts of *C. salina* and *T. chuii* have the most positive records against the fish indicator pathogens (*Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio damsela*, *Vibrio fluvialis* and *Aeromonas hydrophila*). The current study was extended to determine the GC–MS of ethanolic extract of *C. salina* and *T. chuii*. The main constituents detected in the ethanolic extract were organic acids like hexadecanoic acid, octadecanoic acid, and an acyclic diterpene alcohol like phytol.

Introduction

Overuse of antibiotics, appeared to be ineffective in aquaculture and mariculture. It not only increased bacterial resistance, damaged normal microflora of the culture environment causing microdysbiosis, as double pollution; but also caused

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antibiotics residues to accumulate in aquatic products posing risk for human consumption. Counter measures have been taken including Integrated Disease and Pest Management (Li, 2008), especially by using beneficial microorganisms. Sustainable aquaculture or mariculture requires setting up of clean culture models by Basal medium (BM) with no toxin, no side effects, no residue, and no resistance, while it is effective in improving the environment and in raising the immunity of cultured animals, in reducing diseases and in maintaining eco-equilibrium (Bao and Shen, 2005).

As a key cost-saving step, antibiotics are commonly employed in most fish aquacultures to prevent disease. However, the risk in this practice is that antibiotic-resistant pathogens may be spread out along with wastewater to cause serious environmental pollution. To address this problem, an attempt is made to develop a safer, more effective and less expensive biological bactericide for aquaculture use. Documentation is required to evaluate the use of the biocontrol system as an alternative method for inhibition of the fish pathogenic bacteria in infected fish farms (Anne-Marie et al., 2003).

Microalgae are commonly used in the rearing of marine fish larvae. They are either added directly to water in the rearing tanks, when applying the "green water" technique (Reitan et al., 1997), or used as food for rotifers. Addition of microalgae often has a positive effect on the survival rates of fish larvae (Oie et al., 1997). Fishes are susceptible to a wide variety of bacterial pathogens (Schmidt et al., 2000). Many of these bacteria capable of causing disease are considered by some to be saprophytic in nature (Toranzo et al., 2005). These bacteria only become pathogens when fishes are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to proceed (Anderson, 1995).

Antimicrobial activity of microalgae cultures has been shown in earlier studies (Duff et al., 1966; Austin et al., 1992). This antimicrobial activity could be explained by the bacteria present in the microalgae cultures (Dopazo et al., 1988), or by antimicrobial substances produced by the microalgae cells (Marshall et al., 2003; Guedes et al., 2011). KoKou et al. (2012) examined the antimicrobial activity for five cultures of microalgae (*Chlorella minutissima*, *Tetraselmis chuii*, *Nannochloropsis* sp., *Arthrospira platensis* and *Isochrysis* sp.) with no culturable bacteria for testing their ability to inhibit the growth of six *Vibrio* bacterial strains (*Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio scophthalmi*, *Vibrio alginolyticus* and *Vibrio lentus*.

Furthermore, recent advances in algal culture techniques have placed microalgae in a unique position over many other marine organisms, as algae can be cultured under conditions that maximize the production of the desired compound (Chisti, 2007). Thus, the investigation of marine microalgae for their antimicrobial activity is likely to find compounds that can be used in the aquaculture industry. Microalgae are known to produce compounds intracellularly and extracellular, however, as a large proportion of these compounds is not excreted but remains within the cells (Das and Pradhan, 2010; Guedes et al., 2011).

Majority of hatchery protocols in larval rearing systems aim to stop using the addition of algae in the post larval rearing water starting from the age of one month after hatching for saving algae consumption and increasing the cost of their culturing and labor. So, the aims of this study were (i) Determination of microbial profile associated with the cultures of selected microalgae species during 6 weeks of batch cultures, (ii) Evaluation the using different microalgae species as green water on the growth of enriched *Dicentrarchus labrax* post larvae during 6 weeks (iii) Determination of the antagonism effect of different microalga ethanolic extracts in vitro against some pathogenic bacterial strains and identification of the most potent ones using GC–MS.

Materials and methods

Reference bacterial strains

The indicator bacteria used during this work were; *Aeromonas hydrophila*, *Staphylococcus aureus* ATCC 6538, *Vibrio damsela*, *Vibrio fluvialis*, *Streptococcus faecalis* and *Escherichia coli* provided by the culture collection of the Microbiology laboratory, National Institute of Oceanography and Fisheries, Alexandria, Egypt.

Chemicals and media

Ingredients of media were all of analytical grade and obtained from recognized chemical suppliers (mainly Oxoid Co.). Media used for isolation and enumeration of the different bacterial groups were; Seawater agar (Zobell, 1946) for determining viable aerobic heterotrophic bacteria, Mannitol salt agar (Abo-Elela and Farag, 2004) for isolating *Staphylococcus* spp., Thiosulfate citrate bile salt sucrose agar (TCBS) (Kobayashi et al., 1963) for isolating *Vibrio* spp., m-endo-les agar medium (ISO9308/1,1990) for isolation of total coliform, mFC agar medium (ISO9308/1,1990) used for isolation of *E. coli* and m-*Enterococcus* agar medium (ISO9308/2, 1984) used for isolation of *S. faecalis*.

Counting and isolation of bacterial isolates

Serial dilutions from 10^{-2} to 10^{-6} were made using filtered sterilized seawater. A portion (0.1 ml) from each appropriately diluted sample was used to inoculate plates prepared with seawater agar for total bacterial counting. Plates of six selective media were inoculated with 0.1 ml of dilution sample for counting the different bacterial groups: TC (total coliform), EC (*E. coli*), SF (*S. faecalis*), Vibrio spp., *S. aureus* ATCC 6538 and *A. hydrophila*. Purification of bacterial colonies was obtained by streaking a single colony on agar plates of the same medium. The pure colonies obtained were transferred to fresh prepared slants. Sub-cultures were kept at 4°C.

Larvae culture

Four green water techniques plus control (clear water) were carried out using post larvae obtained from eggs derived from induced spawning of *D. labrax* broadstock kept at the marine hatchery of National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. After hatching of eggs, the newly hatched larvae were reared in a conventional way till the post larva stage. Once, the age reached 30 DAH (one month), the

post larvae of sea bass were divided and transferred to 4 groups in triplicates of 200 L cylindro – conical fiber glass tanks. This experiment was concentrated on applying only one variable in the green water technique. Four different microalgae species namely *Nannochloropsis salina* (Eustigmatophyte); *Chlorella salina* (Chlorophyte); *T. chuii* (Parasinophyte) and *Isochrysis galbana* (Chrysophyte) were chosen for this study. The pure strains were obtained from the currently pure culture collections in algal lab. presented in the marine hatchery in NIOF, applied as green water techniques and also for enriching live food (*Artemia* metanuplii) used in post larval rearing period until become juveniles and study their antimicrobial effects on survival, larval development that compared with the control clear water. The daily work was siphoning bottom dirts and only adding the specific alga for each tank without changing any water during the experiment except for the control (formal work of exchanging water was done for control tanks as usual).

The feeding regime

The feeding schedule for the post larvae was by adding enriched *Artemia* metanauplü in density ranging from 5 to 6 individuals' ml^{-1} according to grading age and then the larvae were adapted to feed on pellets starting from day 40 DAH with size (100–200 μm) at (0.1 gm/ 100 L). The enrichment of *Artemia* metanauplii was applied using the same specific microalga for each tank. The algal densities in the rearing tanks were determined according to the cell size of each algal species as follow: (as described in the manual of hatchery production of sea bass and sea bream in the FAO (1999). The light intensity was moderate along the experiment and not direct light (800 lux). The aeration was appropriate and was supplied with an air blower. Mean total length, width, weight, mortality rates of larvae as well as microbiological pathogenic organisms count were accessed weekly for 6 weeks as post larval rearing period.

Algae and live prey culture

Microalgae were mass- cultured and used at peak concentration. Intermediate volumes (2 and 20 L) were used to inoculate 30 L plastic bags and then up scaled in a 200 L transparent fiber glass cylinder at 24 ± 1 °C and a salinity of 35 ± 1g L^{-1} in filtered and sterilized sea water from the Eastern Harbor with F/2 nutritive medium according to Guirllard (1975) for *C. salina* and *N. salina* and the Conway medium as described in Al Ansari (2007) for *I. galbana* and *T. chuii*. *Artemia* cysts were decapsulated and incubated under standard conditions (Sorgeloos et al., 2001) and the newly hatched nauplii were washed and concentrated before use.

Preparation of microalgal extracts

According to Wang et al. (2007), 0.1 g dry powder was mixed with 2 ml absolute ethanol and shaken for 24 h at 20 °C in the dark. The mixture was separated by high-speed centrifugation and the extraction procedure repeated twice. The ethanolic extracts were evaporated to dryness and the residue re-dissolved in 2 ml DMSO stock solution (50 mg/ml).

Antibacterial activity against indicator strains

Fifty milliliters of nutrient agar medium inoculated with indicator microorganisms were poured into all plates. After solidifying, wells were punched out using a 0.5 cm cork borer, and each of their bottoms was then sealed with two drops of sterile water agar. One hundred microliters of tested microalgal extracts were transferred into each well after sterilizing using 0.22 μl sterilized filters. After the incubation period, the radius of clear zone around each well (Y) and the radius of the well (X) were linearly measured in mm. The absolute unit (AU) of each crude extract, which indicates a positive result in the antimicrobial action, was calculated according to the following equation (Yang et al., 1992): $AU = \pi Y^2/\pi X^2$.

Chemical analysis of algal extracts using gas chromatography–mass spectrometry (GC–MS analysis)

The GC–MS analysis was done with standard specification by using GC–MS model Hewlett Packard HB 5890 gas liquid chromatography (GLC) coupled with 5989 B series mass spectrometer (MS). The temperature gradient program was adopted for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was 70 °C and gradually accelerated to 250 °C at a rate of 10 °C per minute. The sample was injected at 250 °C after 18 min. The maximum peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds (Pandey et al., 2010). The concentration of such compounds was calculated by the following formula: Compound concentration percentage = $[P1/P2] \times 100$, where, P1 is the peak area of the compound and P2 is the whole peak area in the fractionated extracts.

Statistical analysis

Data were analyzed and the treatments compared using the a one-way ANOVA with 95% confidence limits ($p < 0.05$) by using SPSS statistical program.

Results

Bacterial pathogens in different algal treatments along six weeks of growth of Dicentrarchus labrax post larvae

During the enrichment period (6 weeks) of *D. labrax* post larvae with different algal species (*T. chuii*, *N. salina*, *C. salina* and *I. galbana*), the microbial profiles were determined (Table 1).

The results showed that, the total bacterial count decreased linearly from the 1st to 6th week in the case of *T. chuii* and *I. galbana* (Table 1). The highest value of total count was recorded at the end of the 1st week with *N. salina* treatment, followed by *I. galbana*. On the other hand, there was a sharp increase in the total bacterial count in the case of the control from 1st to 2th week.

In case of TC, their count was not detected in all treatments except in the 2nd week with the control and *C. salina* (Table 1).

Table 1 Bacterial pathogens in different algal treatments along six weeks of growth of *D. labrax* post larvae.

Algal species	Week	Total bacterial count						
		Total coliform	*Escherichia coli*	*Streptococcus faecalis*	*Vibrio* spp.	*Staphylococcus aureus* ATCC 6538	*Aeromonas hydrophila*	Total count
Control	1	0.0	0.0	64.0 ± 2.0	0.0	3003.0 ± 7.2	0.0	1000.0 ± 10.0
	2	2 ± 1	0.0	0.0	0.0	800.0 ± 10.5	0.0	3317.0 ± 120.0
	3	0.0	0.0	10.7 ± 2.1	151.3 ± 3.2	800.3 ± 11.5	5.7 ± 1.5	2001.7 ± 7.6
	4	0.0	0.0	71.7 ± 4.7	3.7 ± 1.5	53.7 ± 11.9	0.0	1004.7 ± 4.2
	5	0.0	3.0 ± 1.0	34.3 ± 1.5	0.0	351.3 ± 13.1	0.0	189.0 ± 10.5
	6	0.0	0.0	118.0 ± 3.6	270.7 ± 6.03	501.3 ± 4.16	0.0	150.7 ± 4.04
T. chuii	1	0.0	0.0	14.0 ± 3.0*	0.0	1896.7 ± 28.1*	0.0	1251 ± 7.1*
	2	0.0	0.0	0.0	0.0	200 ± 13.0*	0.0	1345 ± 19.9*
	3	0.0	0.0	11.0 ± 2.0	203.0 ± 7.0*	0.0*	30.3 ± 3.5*	909.0 ± 6.6*
	4	0.0	0.0	7.33 ± 1.20*	0.0*	0.0*	0.0	802.7 ± 6.4*
	5	0.0	0.0*	144.7 ± 11*	49.7 ± 3.5*	270.7 ± 6.0*	0.0	299.7 ± 10.5
	6	0.0	0.0	114.0 ± 4.0	9.7 ± 1.5*	50.3 ± 2.5*	0.0	70.3 ± 2.5*
N. salina	1	0.0	33.0 ± 3.0*	0.0*	0.0	3103.0 ± 15.7*	0.0	3493.3 ± 30.6*
	2	0.0	0.0	0.0	0.0	251.0 ± 6.6*	0.0	947 ± 10.8*
	3	0.0	0.0	17.0 ± 5.0	70.0 ± 3.0*	500.3 ± 11.5*	17.7 ± 1.5*	832.3 ± 11.7*
	4	0.0	0.0	4.33* ± 1.5	49.3 ± 3.0*	80.3 ± 2.5*	0.0	571.3 ± 7.1*
	5	0.0	0.0*	26.7 ± 1.5	0.0	303.0 ± 6.1*	0.0	120.3 ± 4.5*
	6	0.0	0.0	349.3* ± 6.03	30.0 ± 3.0*	198.7 ± 4.2*	0.0	250.0 ± 6.0*
C. salina	1	0.0	0.0	4.0* ± 1.0	0.0	3000.7 ± 14	0.0	1346.7 ± 25.2*
	2	40.7 ± 3.1*	0.0	0.0	0.0	799.7 ± 10.5	0.0	1800.0 ± 10.0*
	3	0.0	0.0	75.0 ± 10.0*	150.0 ± 10.0	806.3 ± 25.1	62.3 ± 8.7*	700.0 ± 5.0*
	4	0.0	0.0	40.0 ± 2.0*	2.0 ± 1.0	50.0 ± 5.0	0.0	300.0 ± 10.0*
	5	0.0	0.0*	49.3 ± 3.0*	0.0	348.3 ± 7.6	0.0	100.0 ± 10.0*
	6	0.0	0.0	118.3 ± 4.5	10.3 ± 3.5*	0.0*	0.0	298.3 ± 8.6*
I. galbana	1	0.0	0.0	15.3* ± 2.1	0.0	1999.7 ± 12.5*	0.0	2736.7 ± 51.3*
	2	0.0	0.0	0.0	0.0	101.7 ± 7.6*	0.0	1499.7 ± 12.5*
	3	0.0	0.0	7.30 ± 1.5	198.3* ± 12.6	0.0*	16.3 ± 3.1*	1001.7 ± 17.6*
	4	0.0	0.0	130.3* ± 2.5	0.0*	0.0*	0.0	800.0 ± 15.0*
	5	0.0	0.0*	0.0	0.0	250 ± 5.0*	0.0	500.3 ± 3.5*
	6	0.0	3.0* ± 1.0	0.0	89.0 ± 3.68	249.0 ± 4.6*	0.0	297.3 ± 9.3*

Data presented are the mean ± standard deviation of three replicates.
* Results are significantly different than the control at ($P < 0.05$).

E. coli was not detected almost along the period of experiment (6 weeks) except in only three cases from totally thirty cases. It was recorded at the end of the 1st week with *N. salina*, and in the 6th week in the case of *I. galbana*, but it was recorded with the control and in the 5th week. (Table 1). On contrary, *S. faecalis* was not detected at the end of the 2nd week in all treatments, while it reached its maximum at the end of the 6th week in the case of *N. salina* and *C. salina*. Fish-pathogenic bacteria such as *Vibrio* spp., *Staphylococcus* spp. and *A. hydrophila* were also detected along the period of aquaculture of *D. labra* post larvae. Data in Table (1) confirmed that the *Vibrio spp* was absent absolutely in both 1st and 2nd weeks in all treatments, while it was detected in the 3rd week in all treatments. It had the lowest counts with enrichment with *T. chuii* and *C. salina* at the end of the experiment (Table 1).

In the case of *Staphylococcus* spp., it recorded the highest number during the experiment for all treatments. It was appeared in the 1st week with the highest count in all treatments. It decreased gradually until the end of the experiment, and it disappeared with *I. galbana* and *T. chuii* in the 3rd and 4th weeks, in the 6th week in the case of *C. salina* (Table 1). *A. hydrophlia* was recorded only in all treatments at the end of the 3rd week. It disappeared in the rest of treatments and weeks.

The different growth parameters of D. labrax post larvae at different algal treatments

The main growth parameters of *D. labrax* such as length, weight, width and percentage of survival were estimated at the end of the experiment (after 6 weeks). Data shown in Fig. 1 that the higher length and width of *D. labrax* were recorded in the case of enrichment with *I. galbana* followed by *N. salina*, and its most weight was recorded in the case of *N. salina*, and this increase was significantly different than the control at ($P < 0.05$). The significant increase in percentage of survival of *D. labrax* was recorded in the case of *C. salina* and *T. chuii* (70% and 60.1%, respectively) as compared with the control (22%).

Antibacterial activity (AU) of the different microalgal extracts against some fish indicator pathogens

The antibacterial activity (AU) of the different microalgal ethanolic extracts against some fish indicator pathogens was determined (Table 2). The results presented indicated that the extract of *C. salina* had the most positive records against

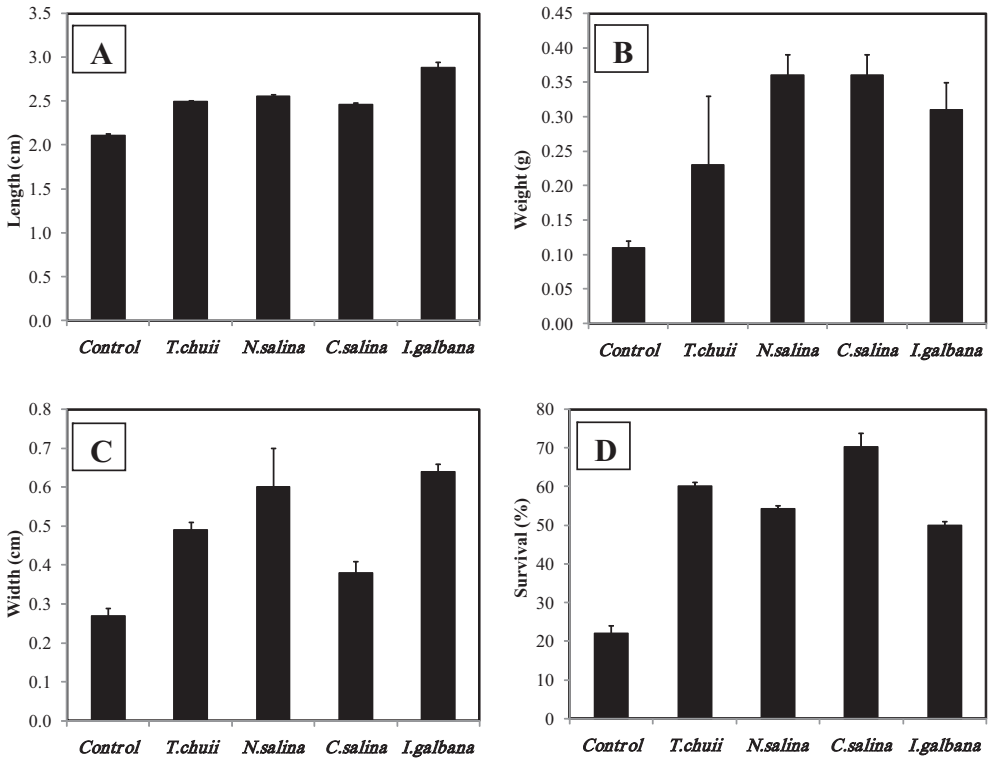

Figure 1 The different growth parameters of *D. labrax*; length (A), weight (B), width (C) and survival% (D) corresponding to different treatments as compared with control after 6 weeks. Data presented are the mean of three replicates, *Results are significantly different than control at ($P < 0.05$).

Table 2 Antibacterial activity (AU) of the different microalgal extracts against some fish indicator pathogens.

Indicator pathogens	Microalgal extract			
	I. galbana	*T. chuii*	*N. salina*	*C. salina*
E. coli	2.4 ± 0.20	5.4 ± 0.30	2.9 ± 0.20	5.4 ± 0.30
P. aeruginosa	–	1.7 ± 0.10	–	2.1 ± 0.10
S. faecalis	2.1 ± 0.0.1	–	1.7 ± 0.0.9	–
S. aureus	–	–	–	–
B. cereus	–	–	2.8 ± 0.20	–
V. damsela	–	4.0 ± 0.20	–	5.4 ± 0.10
V. fluvialis	2.4 ± 0.10	–	–	2.8 ± 0.20
A. hydrophila	–	9.0 ± 0.3.0	–	4.0 ± 0.30

Data presented are the mean ± standard deviation of three replicates.

the fish indicator pathogens especially *E. coli*, *Pseudomonas aeruginosa*, *V. damsela*, *V. fluvialis* and *A. hydrophila*, while the highest AU value occurred by *T. chuii* against *A. hydrophila*. In addition, both extracts of *T. chuii* and *C. salina* had valuable inhibition (AU = 5.4 ± 0.30) against *E. coli*. On the other hand, the extract of *N. salina* had the lowest AU values. It was clearly observed that there was no AU done by all microalgal extracts against *S. aureus*.

MS profile of ethanolic crude extracts of C. salina and T. chuii

The current study was extended to determine the GC–MS of ethanolic extracts of *C. salina* and *T. chuii* which have the highest AU activity against some fish indicator pathogens. The main constituents detected in the GC–MS profile of the two algal species were hexadecanoic acid, octadecanoaic acid and acyclic diterpene alcohol (like phytol). The major constituents were represented in Fig. 2 and Table 3, and their chemical formulae were represented in Fig. 3).

Discussion

The application of the "green water" technique is commonly used in the rearing of marine fish larvae or used as food for rotifers (Reitan et al., 1997).

The numbers of bacteria (total coliforms (TC), *E. coli* (EC) and *Streptococci faecalis* (SF), as well as fish pathogenic bacteria such as; *Vibrio* spp., *Staphylococcus* spp. and *A. hydrophila*)

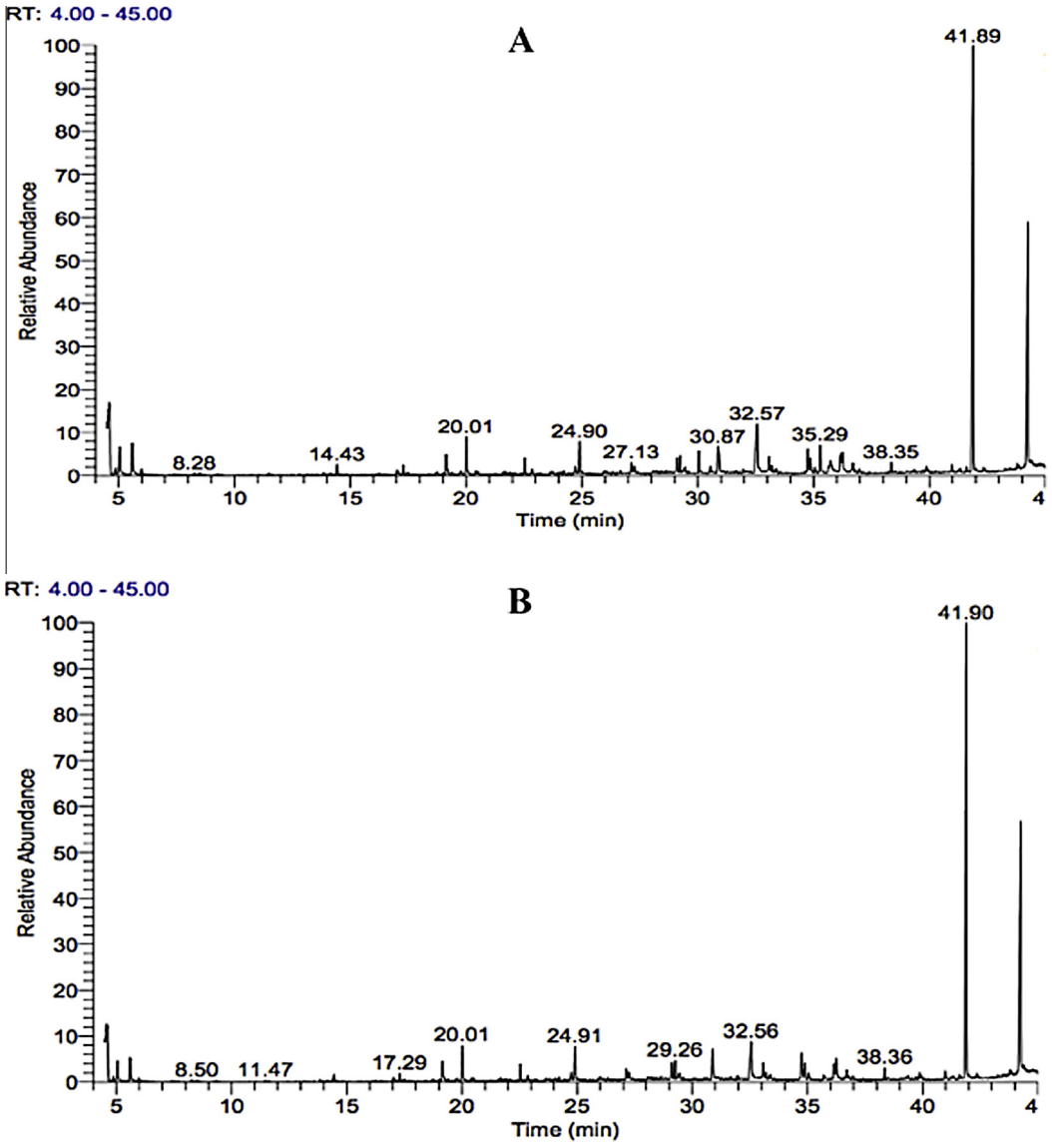

Figure 2 GC–MS profile of ethanolic crude extracts of *C. salina* (A) and *T. chuii* (B).

associated with the microalgae cultures were detected. The obtained results showed that, the total bacterial counts decrease linearly from the 1st to 6th week in the case of *T. chuii* and *I. galbana*. The highest value of total count was recorded at the end of the 1st week with *N. salina* treatment, followed by *I. galbana*, while the control and *T. chuii* had the lowest record. On the other hand, there was a sharp increase in the total bacterial counts in the case of the control from the 1st to 2th week.

Gram et al. (2001) concluded that, many Gram-positive bacterial strains were detected in the cultures; algal species could mean that any possible antimicrobial activity might have been selectively directed against Gram-negative bacterial strains only. On the other side, Ibrahim and El-Shenawy (2008) concluded that the combination of the three fecal indicators is likely to offer a representative tool of water quality.

Düğncl and Candan (2003) reported that *A. hydrophila* is a highly pathogenic bacteria to both cultured and wild fishes, while, the present results showed that, *A. hydrophlia* was recorded only in all treatments with different algal species at the end of the 3rd week. It disappeared in the rest of treatments and weeks. This inhibition may include bactericidal action or competition of other bacterial populations associated with the microalgae or production of antimicrobial substances by the microalgal cells.

The results of KoKou et al. (2012) revealed that the microalgae *C. minutissima*, *T. chui*, *Nannochloropsis* sp., *A. platensis* and *Isochrysis* sp cultures inhibited the growth of *V. parahaemolyticus*, *V. anguillarum*, *V. splendidus*, *V. scophthalmi*, *V. alginolyticus* and *V. lentus* as compared with the control treatments ($P < 0.05$). In the control groups, the number of bacteria increased exponentially during the experimental period in the absence of microalgae cells demonstrating that the bacterial cells were able to utilize the growth medium of microalgae cultures.

The main growth parameters of *D. labrax* such as length, weight and width and percentage of survival were estimated at the end of the experiment (after 6 weeks). Data represented that the most length and width of *D. labrax* were recorded in

Table 3 GC–MS of major components in the *C. salina* and *T. chuii* ethanolic extracts.

Serial No.	Retention Time	Probability	Compound name	Molecular weight	Area %
Chlorella salina					
1	5.05	30.29	Benzene, 1,3-dimethyl-	106.17	0.66
2	14.43	32.09	Dodecane	170.33	0.77
3	17.03	32.80	Naphthalene, 2-methyl-	142.19	0.67
4	17.29	30.83	Tridecane	184.36	0.72
5	20.01	40.49	Tetradecane	198.39	2.66
6	22.53	30.84	Pentadecane	212.41	1.00
7	22.84	56.73	Phenol, 2,4-bis(1,1-dimethylethyl)-	206.32	0.42
8	24.90	25.28	Hexadecane	226.44	2.20
9	32.56	81.03	Hexadecanoic acid (Palmitic acid)	256.42	6.07
10	35.29	80.73	Phytol	296.53	2.36
11	38.35	91.45	Octadecanoic acid, propyl ester	326.56	0.68
12	44.24	98.21	Octabenzone	326.43	19.03
Tetraselmis chuii					
1	5.07	34.31	Benzene, 1,3-dimethyl-	106.17	1.42
2	5.61	27.04	*p*-Xylene	106.17	1.67
3	14.43	32.62	Dodecane	170.33	0.80
4	17.29	30.31	Tridecane	184.36	0.78
5	20.01	33.92	Tetradecane	198.39	2.64
6	22.53	35.13	Pentadecane	212.41	1.16
7	22.84	40.14	Phenol, 2,4-bis(1,1-dimethylethyl)-	206.32	0.48
8	32.56	83.24	Hexadecanoic acid (Palmitic acid)	256.42	5.73
9	34.88	42.63	*n*-Propyl hexadecanoate	298.50	1.11
10	35.29	71.94	Phytol	296.53	0.56
11	38.35	91.86	Octadecanoic acid, propyl ester	326.56	0.70
12	44.24	98.27	Octabenzone	326.43	19.18

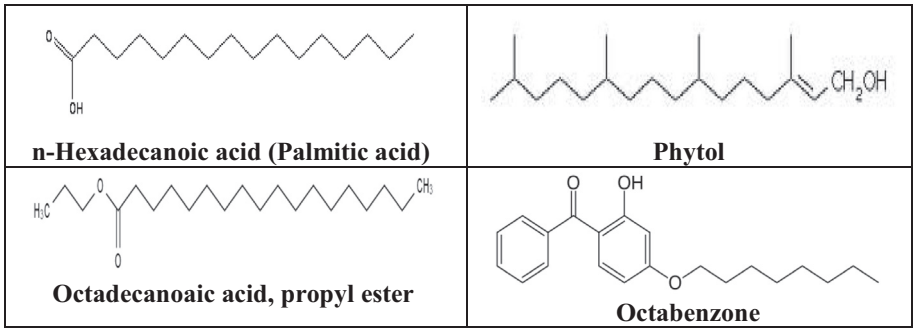

Figure 3 The chemical formula of the major compounds in the mass spectra of ethanolic extracts of *C. salina* and *T. chuii*.

the case of enrichment with *I. galbana* followed by *N. salina*, and its highest weight was recorded in the case of *N. salina*. The highest percentage of survival of *D. labrax* was recorded in the case of *C. salina* (70%) as compared with the control (22%). These results illustrate that the effect of enrichment with the four algal species on the behavior of *D. labrax* post larvae is subtle. These results coincided with the results obtained by Zaki and Saad (2010) who revealed that sea bass larvae attained their maximum growth and survival (3.5 cm and 79%, respectively) by using *C. salina* as enrichment and as a green water treatment. Thus, any improvement in the growth parameters or survival associated with green water is most likely a result of the indirect effects that the microalgae might have on the nutritional quality of their food, and/or on their physiology (Reitan et al., 1997).

The results presented were indicative that the extract of *C. salina* had the most positive records against the fish indicator pathogens especially *E. coli*, *P. aeruginosa*, *V. damsela*, *V. fulvialis* and *A. hydrophila*, while the highest AU value occurred with *T. chuii* against *A. hydrophila*. The results of the present study may explain the low levels or absence of *Vibrio* strains in microalgae cultures, and the positive effect of addition of microalgae in the rearing of fish larvae, and implicate the production of antibacterial compounds by microalgal cells.

In addition, both extracts of *T. chuii* and *C. salina* had valuable inhibition (AU = 5.4) against *E. coli*. There were previously similar observations with the extracts of certain marine algae against *E. coli*, *P. aeruginosa*, *S. aureus* (Ananatharaj et al., 2004; Chandrasekaran et al., 2005).

Also, the current study showed that *C. salina* and *T. chuii* exhibited higher antimicrobial features against the strongest pathogen for sea bass larvae (*Vibrio damsela*) with AU values of 5.4 and 4.0, respectively. The same achievements were obtained for *C. minutissima* and *T. chuii* studied by Makridis

et al. (2006). Thus the results of this study demonstrate a simple and practical approach to decrease the microbial load and at the same time may reduce the percentage of *Vibrio* among the bacteria associated with enriched *Artemia*.

Austin et al. (1992), also, reported that, *Tetraselmis suecica* when used as a food supplement for fish, the algal cells inhibited laboratory induced infection in Atlantic salmon. Li and Tsai (2009) attempted to culture *Nannochloropsis oculata* in a way that would provide an organism against pathogenic infections.

The obtained results of GC–MS revealed the presence of Hexadecanoic acid (Palmitic acid) (6.07 and 5.73%) in the ethanolic extract of *C. salina* and *T. chuii*, respectively which have antimicrobial activity. Other constituents were detected in ethanolic extracts of *C. salina* and *T. chuii* such as; organic acids (like octadecanoaic acid) and acyclic diterpene alcohol (like phytol); which is an acyclic diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamins E and K1 (Netscher, 2007), also it is a constituent of chlorophyll, which is then converted to phytanic acid and stored in fats in some animals (Brink and Wanders, 2006). Indirect evidence has been provided that significant amounts of phytol came from the hindgut fermentation of plant materials (Watkins et al., 2010; Moser et al., 2013). The present data collected on the antibacterial activity of such compounds matched with hypotheses of other workers (Abo-Elela et al., 2009; Manilal et al., 2009; Ibrahim and Abd El-Naby, 2010; Ibrahim, 2012; Ibrahim et al., 2012).

In accordance to our study, Agustini (2009) concluded that *T. chuii* have bioactive components from fatty acid and ester groups which can be used as antibacterial agents that are capable of inhibiting the growth of *E. coli* and *S. Aureus*. Lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic acids are known to have potential antibacterial and antifungal agents; Seidel and Taylor, 2004; Agoramoorthy et al., 2007). Many fatty acids are known to have antibacterial and antifungal properties (Russel, 1991).

The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Mayer and Hamann, 2005; Cardozo et al., 2007; Agustini, 2009) for instance, the antimicrobial activity of supercritical extracts obtained from the microalga *Chaetoceros muelleri* was related to its lipid composition (Mendiola et al., 2007).

Jaya et al. (2013) showed that the bioactive compounds present in *T. chuii* extract were fatty acids, esters, alcohols, ketones, benzene, and alkanes. The functionality of extracellular microalgal products act as inhibitors and trigger growth compounds. *T. chuii* has bioactive components such as fatty acids and esters and are used as antibacterial agents.

Bai and Krishnakumar (2013) showed that methanol plus chloroform (1:1) crude extract of *T. suecica* showed confirmed considerable activity against gram negative bacteria than gram positive human pathogens. GC–MS analysis revealed the presence of unique chemical compounds like 1-ethyl butyl 3-hexyl hydroperoxide (MW: 100) and methyl heptanate (MW: 186) respectively for the crude extract of *T. suecica*. Different fatty acids such as Methyl caproate, Methyl stearate, Decoic acid, Palmitic acid, Nonoic acid and Caprylic acid with antimicrobial activity and pharmaceutical importance were identified.

Similar results were obtained by Santoyo et al. (2009) who reported that, ethanol extracts from *Haematococcus pluvialis* possess antimicrobial activity against a Gram-ve bacterium, *E. coli*, and a Gram + ve bacterium, *S. aureus*; this was once again associated with the presence of short-chain fatty acids, namely butanoic and methyl lactic acids. Results from in vitro experiments cannot, however, be extrapolated to explain the microbial conditions found in the different cultures of *D. labrax* post larvae. It has been shown that such inhibition in vitro of pathogens does not necessarily correspond to any significant effect in routine culture conditions (Gram et al., 2001).

Conclusions

Our study undoubtedly confirms the following points:

- The "green water" technique is applied here successfully in the rearing of *D. labrax* larvae.
- Marine microalgae are known to produce bioactive compounds both intra-cellular and extra-cellular. So, they could be applied as an alternative for antibiotics that are commonly employed in most fish aquacultures to prevent disease.
- The counting of bacteria (total coliform, *E. coli* and *S. faecalis*, as well as fish pathogenic bacteria such as; *Vibrios*, *Staphylococci* and *A. hydrophila*) associated with the microalgae cultures was shown in low counts or disappeared in the different algal treatments (*I. galbana*, *N. salina*, *C. salina* and *T. chuii*).
- The main growth parameters besides survival percentage of *D. labrax* were improved with the different treatments as compared with the control.
- Using of the two microalgae; *C. salina* and *T. chuii* for the enrichment of *D. labrax* post larvae contain higher relative percentage of the palmitic acid and other bioactive substances that have potential antimicrobial activities.

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