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Phylogenetic diversity of culturable bacteria in *Chaetoceros gracilis* mass culture system of a marine finfish hatchery

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Original Article

Abstract

Microalgae, a major live feed in aquaculture always coexist with associated bacteria. Hence a better understanding of algal-bacterial interaction is essential for maintaining a stable environment in intensive larval rearing tanks. Therefore, herein we attempted to determine the phylogenetic diversity of culturable bacteria associated with microalgal production system of a marine finfish hatchery with special reference to *Chaetoceros gracilis* mass culture. The sequencing of 16S rDNA of representative from each phylotypes revealed that the associated microflora belong to the classes *Gammaproteo bacteria, Alphaproteo bacteria,* and *Bacilli*. In particular, members of *Marinobacter* genus showed higher degree of association followed by *Leisingera, Alteromonas, Nautella, Halomonas* and *Ruegeria.* The association of bacterial groups belonging to the genera *Idiomarina, Albidovulum* and *Staphylococcus* were also detected. The variation of bacterial diversity in microalgal habitat with changes in environmental conditions was also discussed in the present work. In overall, the present study gives a greater insight to the algal microhabitat which would be vital for improving stability, productivity, sustainability and reliability of large scale microalgal cultivation and their feeding to the target aquaculture species.

Keywords *: Algal-bacterial association, live feed, culturable bacteria, aquaculture, molecular phylogeny*

Introduction

Aquaculture is the fastest growing food-producing sector in the world and it is estimated that 44.14 % of fish produced globally is contributed by aquaculture (Dauda *et al*., 2018). Microalgae are ideal candidates as major live feeds in aquaculture, especially in larval rearing systems, due to their characteristics such as high nutrient content, rapid growth rate, non-toxicity, appropriate size for ingestion and digestion, stability and sustainability of mass culture etc. (Salvesen *et al*., 2000; Flandez, 2011). Other than the nutritional support, these microalgal live feeds may have an impact on bacterial communities of the rearing tanks since they always coexist with bacteria in natural aquatic ecosystem (Salvesen *et al*., 2000; Guo and Tong, 2014). Our previous study clearly confirmed the presence of diverse bacterial groups in microalgal habitat and the concentration of culturable bacteria varied from 101 to 105 CFU mL^{-1} of algal culture (Sandhya *et al*., 2017). According to Nicolas *et al.* (2004) the algal cultures were associated with more

number of bacteria than sea water and their impact on larvae may depend on their concentration. These bacterial counterparts might greatly improve the nutritional quality of rearing animal since they can enhance growth and chemical composition of phytoplankton host (Natrah *et al.*, 2014; Fuentes *et al.*, 2016). For example, Toi *et al.* (2014) reported the production of healthier *Artemia* cultures through the co-ingestion of algae and bacteria. Thus the interaction between microalgae and bacteria play a key role in productivity and sustainability of aquaculture (Natrah *et al.*, 2011). Moreover, results of our earlier study suggested the potential of these associated bacteria in preventing the invasion of pathogenic bacteria in algal habitat by competitive exclusion (Sandhya *et al.*, 2017). In addition to these beneficial aspects, inhibitory effects of associated bacteria on algal growth and metabolism were also reported (Cole, 1982; Natrah *et al.*, 2014; Fuentes *et al.*,2016). Thus in order to determine the impact of these associated bacteria on the microbial environment in aquatic hatcheries, the first step is to study the diversity of microalgal bacterial flora (Nicolas *et al.*, 2004). The chemical composition of microalgae varies with the changes in physical and chemical environment and it may also have an influence on the growth of associated bacterial communities (Salvesen *et al.*, 2000). Hence, better understanding of the phycosphere niche is highly relevant for improving the cultivation process of microalgae used as feed in aquaculture. In this context, the present work aims to study the phylogenetic diversity of culturable bacteria associated with the microalgal production system of a marine finfish hatchery and to assess whether environmental factors have an influence on microflora of microalgal habitat.

Material and methods

Sample collection

The microalgae (*Chaetoceros* sp.) culture samples from various stages of mass culture *i.e.,* from 250 mL flask, 1L flask, 10 L cylinder, 100 L outdoor tank, 500 L outdoor tank and 2 ton outdoor tank were collected in every three month interval for a period of one year during March 2013 to December 2013 from a marine finfish hatchery at Alappuzha, Kerala, India (West Coast Hatcheries & Research Centre Pvt Ltd.). The same microalgal strain was maintained in the microalgae culture collection of the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India) as strain *'Chaetoceros gracilis* MBTD-CMFRI-S172', after morphological and molecular identification (18S rRNA gene sequence similarity; GenBank Acc No: KM087981).

Isolation and identification of associated bacteria

The isolation and identification of bacteria associated with microalgae was carried out as described in Sandhya *et al.*, 2017. In brief, 10 ml of microalgal culture from different stage of mass culturing was filtered through a 1.2 μm membrane filter (Pall) and the filter cake obtained was washed three times with 0.85% sodium chloride (NaCl). It was then vortexed, serially diluted and plated on both Zobell Marine Agar (ZMA) (Himedia, India) and thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia, India). Morphologically different colonies grown on ZMA plates were selected for further purification and preservation.

The total genomic DNA was extracted from all bacterial isolates by phenol-chloroform enzymatic extraction method and 16S rDNA from the genomic DNA was amplified by PCR with universal primers NP1F (5'-GAG TTT GAT CCT GGC TCA-3') and NP1R (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Sambrook and Russell 2001; Pai *et al.*, 2010). The PCR reaction was carried out as described by Nair *et al.* (2012) in Veriti thermal cycler (Applied Biosystems, Germany). After purification (HiPura PCR product purification kit, Himedia), the amplified PCR products were sequenced by Sanger sequencing method. The isolated bacterial strains were identified upto generic level based on their 16S rDNA sequence similarity with the sequence available in EzTaxon database (Kim *et al.*, 2012).

Phylogenetic analysis

The molecular phylogeny was inferred using neighbour-joining method (Saitou and Nei, 1987). CLUSTALW algorithm was used for multiple alignment and evolutionary analyses were conducted in MEGA6 (Thompson *et al.*, 1994). The tree topologies were evaluated by bootstrap analysis of 1000 data sets and evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Since the sequencing reaction of six isolates (WC 01, WC 32, WC 36, WC 39, WC 40, WC 46) repeatedly failed with the primer NP1F, only NP1R was used to sequence their 16S rDNA genes. Similarly, for the isolate WC 59 the primer NP1F alone was used. Hence their sequences were not included in the phylogenetic analysis. All 16S rDNA sequences were submitted to the NCBI GenBank.

Bacterial diversity analysis

Bacterial diversity was measured by calculating Simpson reciprocal diversity index. It was defined as $1/\sum n(n-1)/N(N-1)$ where n is the number of organisms of a particular genus and N is the number of organisms of all genera (Suchodolski *et al.*, 2008).

Results and discussion

In the present study, isolation and identification of culturable bacteria associated with the microalgal production system of a marine finfish hatchery was undertaken. For bacterial isolation, Zobell Marine Agar was used which is previously reported as reference medium to study bacterioplankton (Nicolas *et al.*, 2004; Lebaron *et al.*, 2001). After incubation, growth of diverse subsets of bacteria was found on ZMA inoculated with microalgal cultures.

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Totally, 69 bacterial isolates were obtained (Strain code WC 01- 14, 19-73; Table 1) and their 16S rDNA sequences shared 88-100 % similarity with known bacterial genera in EzTaxon database. The molecular identification revealed that they showed maximum similarity to the genera *Marinobacter, Leisingera*, *Nautella, Alteromonas, Idiomarina*, *Halomonas*, *Albidovulum, Ruegeria* and *Staphylococcus*. A neighbour-joining phylogenetic tree constructed with their 16S rDNA sequences separated the obtained bacterial isolates into three different clades as *Gammaproteo bacteria* (78.26%), *Alphaproteo bacteria* (20.29%) and *Bacilli* (1.45%) (Fig. 1). The class *Gammaproteo bacteria* comprise 54 isolates belong to four different genera *Marinobacter, Alteromonas, Idiomarina* and *Halomonas*. 14 bacterial isolates belong to the genera *Nautella, Albidovulum, Leisingera* and *Ruegeria* were documented from the class *Alphaproteo bacteria*. From the class *Bacilli* only one isolate namely *Staphylococcus* sp. was obtained.

Table 1. Identification of culturable bacteria associated with microalgal production system using 16S rDNA sequence data. Strain codes for all bacterial isolates starts with MBTD CMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India).

Month	Stage of mass culturing	Strain Code	GenBank accession no.	Phylogenetic group	Similarity (%)
March	250 ml	WC 01	KU572438	*Marinobacter* sp.	99.86
		WC 02	KU554452	*Marinobacter* sp.	97.49
	1 L	WC 03	KU554453	*Marinobacter* sp.	97.45
		WC 04	KU554454	*Marinobacter* sp.	100
		WC 05	KU554455	*Leisingera* sp.	98.03
	10 L	WC 06	KU554456	*Marinobacter* sp.	97.45
		WC 07	KU554457	*Marinobacter* sp.	100
	100 L	WC 08	KU554458	*Marinobacter* sp.	100
	500 L	WC 09	KU554459	*Marinobacter* sp.	97.45
		WC 10	KU554460	*Marinobacter* sp.	100
		WC 11	MF991457	*Alteromonas* sp.	90.50
		WC 12	KU554461	*Nautella* sp.	100
	2 Ton	WC 13	MF991458	*Alteromonas* sp.	90.50
		WC 14	KU554462	*Nautella* sp.	100
June	250 ml	WC 54	KU554496	*Marinobacter* sp.	99.28
		WC 55	KU554497	*Marinobacter* sp.	99.14
		WC 56	KU554498	*Alteromonas* sp.	99.78
		WC 57	KU554499	*Marinobacter* sp.	99.93
	1 L	WC 58	KU554500	*Idiomarina* sp.	98.77
		WC 59	MF991460	*Halomonas* sp.	98.86
	10 L	WC 60	KU554501	*Leisingera* sp.	98.23
		WC 61	KU554502	*Marinobacter* sp.	99.85
		WC 62	KU554503	*Marinobacter* sp.	99.14
		WC 63	KU554504	*Marinobacter* sp.	99.86
	100 L	WC 64	KU554505	*Marinobacter* sp.	100
		WC 65	KU554506	*Marinobacter* sp.	97.59
	500 L	WC 66	KU554507	*Marinobacter* sp.	100
	2 Ton	WC 67	KU554508	*Marinobacter* sp.	97.5
		WC 68	KU554509	*Marinobacter* sp.	97.64
		WC 69	KU554510	*Idiomarina* sp.	100
		WC 70	KU554511	*Marinobacter* sp.	99.86
		WC 71	KU554512	*Marinobacter* sp.	99.64
September	250 ml	WC 19	KU554466	*Alteromonas* sp.	99.71
		WC 20	KU554467	*Nautella* sp.	100
		WC 21	KU554468	*Albidovulum* sp.	99.93
	1 L	WC 22	KU554469	*Marinobacter* sp.	100
		WC 23	KU554470	*Marinobacter* sp.	100
		WC 24	KU554471	*Marinobacter* sp.	100
	10 L	WC 25	KU554472	*Marinobacter* sp.	100
		WC 26	KU554473	*Marinobacter* sp.	99.93
	100 L	WC 27	KU554474	*Marinobacter* sp.	99.93
		WC 28	KU554475	*Marinobacter* sp.	100
		WC 29	KU554486	*Staphylococcus* sp.	99.93
	500 L	WC 30	KU554487	*Marinobacter* sp.	100
		WC 31	KU554488	*Leisingera* sp.	98.25
		WC 32	MF991459	*Marinobacter* sp.	88.61
	2 Ton	WC 33	KU554489	*Leisingera* sp.	98.11
		WC 34	KU554490	*Marinobacter* sp.	97.27
		WC 35	KU554491	*Marinobacter* sp.	100
December	250 ml	WC 36	KU572440	*Marinobacter* sp.	99.53
		WC 37	KU554492	*Ruegeria* sp.	99.92
		WC 38	KU554493	*Idiomarina* sp.	97.29
	1 L	WC 39	KU572441	*Leisingera* sp.	99.01
		WC 40	KU572442	*Leisingera* sp.	99.48
	10 L	WC 41	KU554494	*Marinobacter* sp.	99.93
		WC 42	KU554495	*Nautella* sp.	100
		WC 43	KU554476	*Marinobacter* sp.	99.79
		WC 44	KU554477	*Marinobacter* sp.	99.71
	100 L	WC 45	KU554478	*Marinobacter* sp.	99.93
		WC 46	KU572443	*Leisingera* sp.	98.94
		WC 47	KU554479	*Marinobacter* sp.	99.86
		WC 48	KU554480	*Marinobacter* sp.	99.93
	500 L	WC 49	KU554481	*Marinobacter* sp.	100
		WC 50	KU554482	*Marinobacter* sp.	99.77
		WC 51	KU554483	*Nautella* sp.	100
		WC 52	KU554484	*Marinobacter* sp.	99.93
		WC 53	KU554485	*Marinobacter* sp.	98.42
	2 Ton	WC 72	KU554513	*Marinobacter* sp.	97.87
		WC 73	KU554514	*Marinobacter* sp.	97.18

The microalgal suspensions were also inoculated on TCBS agar plates in order to determine whether any pathogenic bacterial groups like *Vibrio* spp. were associated. It was reported that sometimes the microalgae might stimulate the growth of pathogens and it can exert an overall negative effect to the aquaculture production system (Natrah *et al.*, 2014). Also, Gomez-Gil *et al.* (2002) observed better growth of aquaculture pathogen like *Vibrio alginolyticus* in the presence of *Chaetoceros muelleri*. But in contradict to the above observations, there was no bacterial growth on TCBS plates which indicated the absence of *Vibrio* spp. As suggested by Santos and Reis (2014) it may be due to the competitive exclusion by phycosphere bacteria and our results confirms the safety of using this live feed in larval rearing systems.

Previously, we obtained bacterial groups belonging to four different genera *Marinobacter*, *Oceanicaulis*, *Labrenzia*

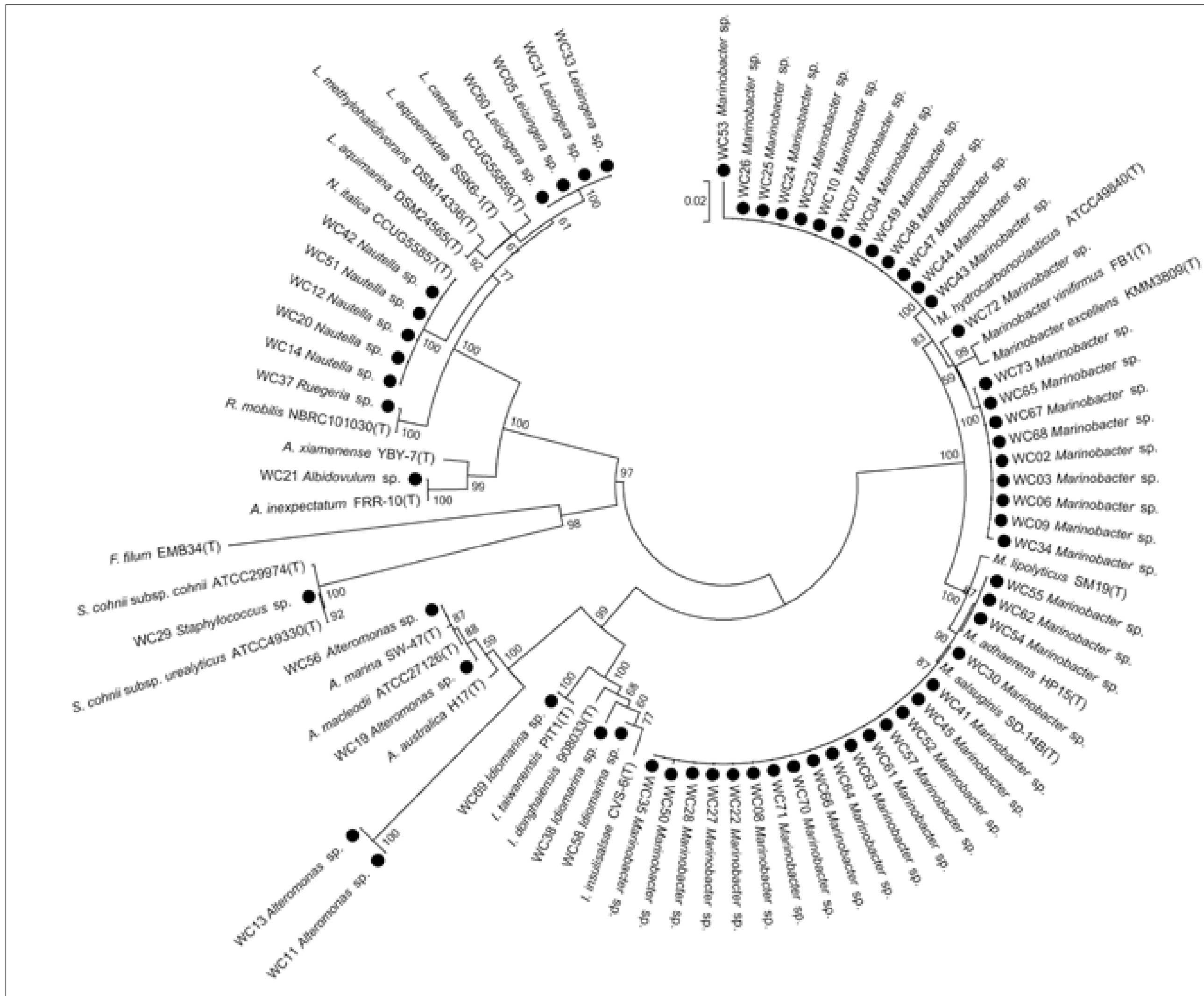

Fig. 1. Neighbour-joining phylogenetic tree based on partial 16S rDNA sequence of culturable bacterial strains isolated by this study and reference strains from the EzTaxon database. Strain codes for all bacterial isolates starts with MBTD CMFRI (not shown) to indicate they were obtained at the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Cochin (Kerala, India)

and *Alteromonas* from laboratory maintained culture of *Chaetoceros* sp. (MBTDCMFRI S065, GenBank Acc No. JF708154) (Sandhya *et al*., 2017). In the present study *Marinobacter* spp. were obtained from most stages of microalgal production system throughout the year (66.67 %). Similarly the association of *Alteromonas* spp. was also observed except in the month of December. There are many reports which support our isolation of these bacterial genera from microalgal culture (Jasti *et al*., 2005; Sapp *et al*., 2007; Ali *et al*., 2010; Amin *et al*., 2012; Le Chevanton *et al*., 2013; Natrah *et al*., 2014). At the same time, neither *Labrenzia* nor *Oceanicaulis* were obtained from any stages of the mass culturing of selected *Chaetoceros* sp. In addition to *Marinobacter* and *Alteromonas*, seven other bacterial groups (*Leisingera, Nautella* , *Idiomarina, Halomonas, Albidovulum, Staphylococcus* and *Ruegeria*) were found to be associated with different stages of *Chaetoceros gracilis* production system. Bacterial groups isolated during each sampling were shown in Fig. 2. The genus *Leisingera* is a member of *Roseobacter* clade within the family *Rhodobacteraceae*. They are reported to be present in various marine habitats including symbiosis with algae (Vandecandelaere *et al*., 2008; Riedel *et al*., 2013). Similarly Oh *et al.* (2011) observed the association of *Nautella* sp. with marine dinoflagellate *Cochlodinium polykrikoides*. Likewise, Porsby *et al.* (2008) supported our isolation of *Ruegeria* sp. from microalgal production system. In addition to that Arora *et al.* (2012) documented close association of three bacterial strains including *Ruegeria* sp. with marine microalgae *Tetraselmis indica*. Also, *Halomonas* sp. identified from our study was previously documented to be associated with microlage *Alexandrium minutum* (Palacios *et al*., 2006). However, to

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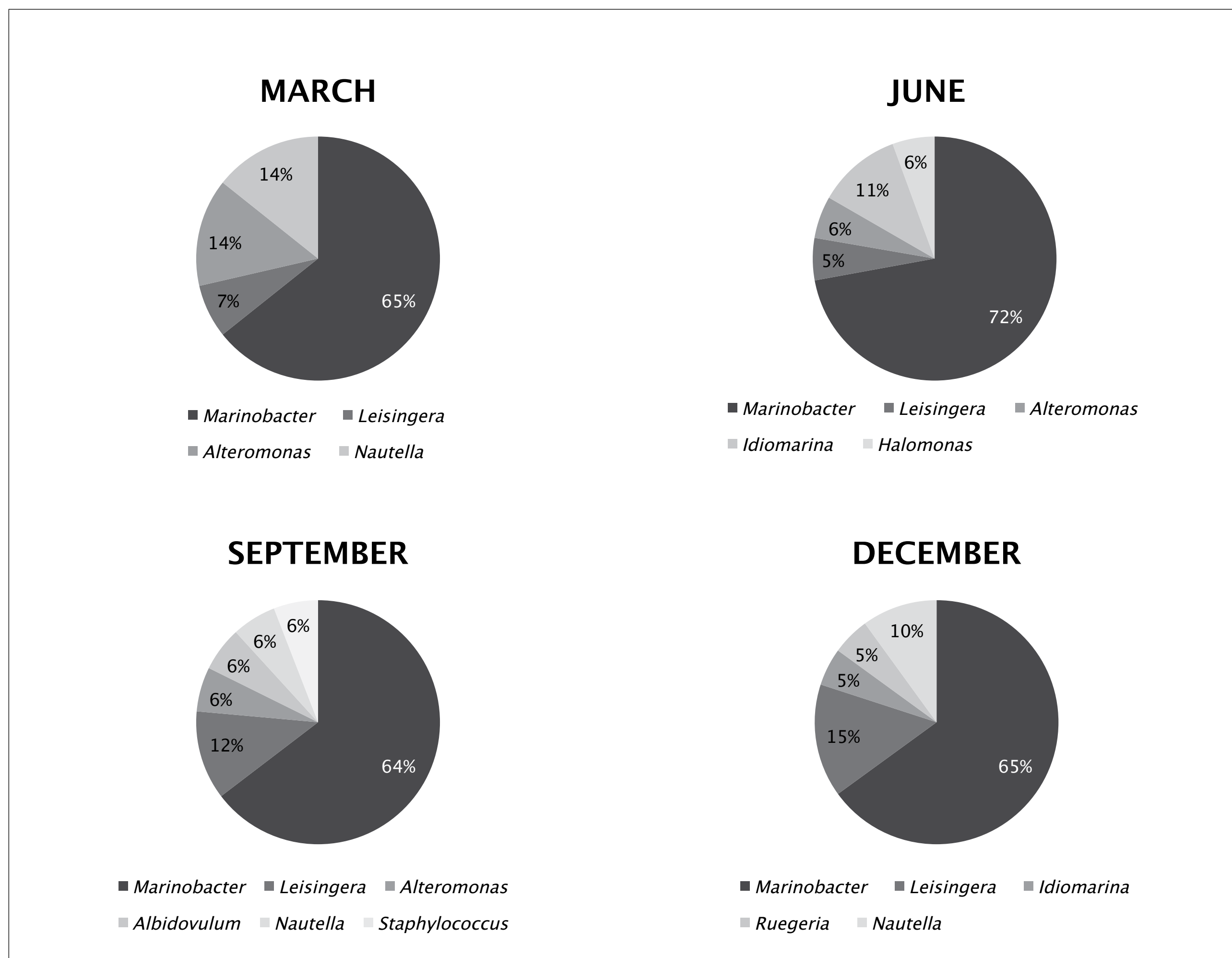

Fig. 2. Variation of culturable bacterial diversity in microalgal production system during each sampling.

the best of our knowledge it is the first report on microalgal association of bacterial groups belonging to the genera *Idiomarina, Albidovulum* and *Staphylococcus*.

It was observed that *Marinobacter* spp. were predominantly present in the selected microalgal production system which clearly indicated a close association of this bacterial genus with the selected strain of *Chaetoceros gracilis*. However, association of other bacterial groups showed considerable variation in each sampling. Bacterial diversity was measured by calculating Simpson reciprocal diversity index and was shown in Table 2. Simpson reciprocal diversity index yield information about bacterial diversity and high value for the index indicate high bacterial diversity (Suchodolski *et al*., 2008). The maximum bacterial diversity was obtained in the month of September followed by March, December and June. The results suggested that there is a variation in bacterial diversity with changes in physical and chemical factors. This was found to be in agreement with one previous study which reported that bacteria – phytoplankton interactions are highly variable with environmental conditions (Grossart, 1999). It may be due to the changes in the chemical composition of microalgae with varying environmental conditions (Reitan *et al*., 1994; Salvesen *et al*., 2000). Thus our results indicated that the chemical microenvironment created by phytoplankton host might have an influence on the growth of associated bacterial community. Also, the phycosphere bacteria may be influenced by the algal cell number and growth conditions which could vary considerably between sampling (Salvesen *et al*., 2000).

Table 2 Simpson reciprocal diversity index (1/D) of each sampling

Month	Simpson reciprocal diversity index (1/D)
March	2.39
June	1.95
September	2.43
December	2.32

In summary, the present study revealed that microalgal production system of aquatic hatcheries was associated with

diverse bacterial groups. Microalgae represent a repetitive source of bacterial inoculation into the larval rearing tanks since they are added at regular intervals into the system to maintain specific algal density (Salvesen *et al.*, 2000). This repetitive inoculation of bacteria through microalgal addition might have a significant effect on the microflora of water and larvae. Makridis *et al.* (2006) reported that the bacteria associated with the live feed play an important role in the exponential proliferation of bacteria in the fish gut during the early development of the larvae. Also, these associated bacterial flora often result in elimination of contaminating bacteria in aquaculture system through competitive exclusion (Santos and Reis, 2014; Fuentes *et al.*, 2016). Thus microalgae associated bacteria can outcompete the pathogens and could have a positive impact on aquaculture disease control (Natrah *et al.*, 2014; Fuentes *et al.*, 2016). On the whole, it is clear that enhanced larval growth and development is attributed by the high nutritional value of the live feed as well as by the algae-bacteria interactions (Skjermo and Vadstein, 1993). Also, a greater insight on algal microhabitat is essential for developing a pathogen-free hatchery rearing system. This work is an attempt to improve our knowledge on algal-bacterial interaction which could be vital for successful hatchery larval rearing. Several novel bacterial isolates adapted to the life in the phycosphere of microalgae used in aquaculture systems were identified. In addition, it was found that the chemical microenvironment created by the phytoplankton host might have an impact on diversity of bacteria present in algal habitat. Future research may consider the effect of these interactions in larval growth and development. Thus the gathered information can be further explored for developing a suitable consortium of bacteria that have wide spectrum applicability in aquaculture.

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