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# Evaluation of relationship between Chilean octopus (*Octopus mimus* Gould, 1852) egg health condition and the egg bacterial community

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## Abstract

The objective of this study was to evaluate how bacterial community associated with Chilean octopus (*Octopus mimus*) egg was related to egg health condition using a culture dependent method and PCR-DGGE fingerprinting technique. Total heterotrophic bacterial number of fresh egg was much lower than infected egg. However, biodiversity of culturable bacterial community associated with the fresh egg exhibited a higher diversity than the infected egg. Result of a culture dependent method showed that *Roseobacter* clade was predominant in the fresh egg, while predominant species in the infected egg was γ-proteobacteria. DGGE fingerprinting technique showed that fresh egg associated unculturable bacterial community was constituted of *Roseobacter* clade and Bacteroidetes, whereas Bacteroidetes was predominant bacteria in the infected egg. These results suggest that there might be some sort of relationship between octopus eggs associated bacterial community and egg health condition. Moreover, *Roseobacter* clade and Bacteroidetes might be potential symbiotic bacteria associated with the octopus egg, and some γ-proteobacteria might be involved in octopus egg disease. In particular, *Roseobacter* clade may play an important role in octopus egg health and it raises the possibility that this clade can be utilized as potential probiotics for octopus aquaculture.

**Keywords:** bacterial community, octopus egg, *Octopus mimus*, PCR-DGGE, *Roseobacter* clade

## Introduction

There is worldwide commercial interest in octopus aquaculture. Octopus is an important resource for artisanal benthic fishing ground in Chile (Rocha & Vega 2003). At present, octopus fishery in Chile is based primarily on two species, northern octopus (*Octopus mimus*) and southern octopus (*Enteroctopus megalocyathus*). There is lack of knowledge on reproduction aspect and larval development of cephalopod including octopus. Recently, investigation of octopus egg demonstrated an increasingly trend. Octopus eggs were laid on female arms inside the dens to protect them from external environment during incubation (Rocha, Guerra & Gonzalez 2001). Uriarte, Iglesias, Domingues, Rosas, Viana, Navarro, Seixas, Vidal, Ausburger, Pereda, Godoy, Paschke, Farías, Olivares and Zuñiga (2011) suggested that understanding the influence of environmental factor such as temperature on the rate and efficiency of *Octopus vulgaris* egg yolk utilization were important for early development, growth and survival of paralarvae as well as other cephalopods. Subsequently, they suggested that development and characteristic of *Octopus mimus* egg was affected by culturing temperature (Uriarte, Espinoza, Herrera, Zúñiga, Olivares, Carbonell & Rosas 2012). González, Arriagada, López and Pérez (2008) suggested that improvement of the culture conditions during the embryonic development stage was needed, since egg mortality of baby octopus *Robsonella fontanianus* (d'Orbigny 1834) occurred when egg was contaminated after detached from female.

Hansen and Olafsen (1999) indicated that bacterial adhesion and colonization of the egg surface occurred within hours after fertilization. Moreover, they suggested that species-specific adhesion to the egg surface may play a role in the development of the egg epiflora, although the diverse flora which eventually developed on the egg surface reflected to the environmental water bacterial community. Olafsen (2001) suggested that a dense, nonpathogenic and diverse egg epiflora may be a barrier against colony formation by pathogens. Until now, there are some reports about bacteriological studies of egg associated with marine animals (Hameed 1997; Phatarpekar, Kenkre, Sreepada, Desai & Achuthankutty 2002; Al-Bahry, Mahmoud, Elshafie, Al-Harthy, Al-Ghafri, Al-Amri & Alkindi 2009; Leyton & Riquelme 2010; Mickėnienė & Šyvokienė 2011). Kennedy, Venugopal, Karunasagar and Karunasagar (2006) showed that high rate of *Vibrio* spp. was presented in the egg and larvae of freshwater prawn, but their number was decreased as growth processed. They also suggested that crustacean egg were axenic, but colonized by bacteria when released into the environment. Leyton and Riquelme (2010) suggested that the presence of bacteria in the egg of *Concholepas concholepas* (common name 'Loco') was attributed to vertical transmission from their parents. Moreover, Leyton, Varas-Psijas and Riquelme (2012) indicated that egg associated bacteria could be used as a probiotics in aquaculture, since loco larvae supplemented with some bacteria isolated from loco egg had higher survival rates than control. On the other hand, microbiology information on octopus egg was scarce comparing with data on other marine animals. Uriarte *et al.* (2011) showed that after 15 days spawning the egg of *Enteroctopus megalocyathus* was colonized by bacteria, although there were no bacteria on the egg surface at spawning. This suggested that egg microbiota composition was highly influenced by culture water. Moreover, the infected egg changed colour from whitish to yellow, bacterial number was significantly higher than healthy ones and many filamentous bacteria were found. However, their study did not suggest the different composition of bacterial community between healthy and infected egg sample. Furthermore, information on the microbiological monitoring of other octopus egg is still scarce. Therefore, the present study investigated and compared the bacterial community composition of Chilean octopus (*Octopus mimus*) egg under different health condition (fresh or infected octopus egg) using culturable method and denaturing gradient gel electrophoresis (DGGE) fingerprinting technique. This information could be useful for understanding correlation between bacterial community and the health condition of farmed octopus egg, as well as for usage of probiotics to control bacterial community and improve the octopus egg growth.

## Materials and Methods

### Sample collection

All egg samples were derived from the same spawning time obtained from Unit of Recirculation Larvae Culture at University of Antofagasta in March 2012. We distinguished between fresh and infected egg as described by Uriarte *et al.* (2011). Fresh and infected eggs ($0.1 \pm 0.02$ g each egg cluster, $n = 3$) were collected separately and rinsed three times by shaking in sterile PBS to remove loosely attached cells and debris. Thereafter, each sample was transferred to sterile plastic bags with sterilized PBS and homogenized in a Stomacher Lab-Blender 80 (Tekmar, NY, USA), kept on ice and analysed within 2 h of collection for bacterial enumeration and isolation or moved to frozen storage at −30°C for molecular analysis.

### Enumeration, isolation and 16S rDNA amplification of culturable bacterial community

For enumeration of heterotrophic bacteria, determination of relative abundance and species composition of culturable microorganisms, each homogenate sample was diluted in sterile ASW up to $10^{-5}$ dilution. Thereafter, 0.1 mL of each dilution was spread onto ZoBell 2216E agar medium and incubated at 20°C for 7 days. After incubation, plates with 30 to 300 colonies were counted and the statistical significance of the difference between the data of the two groups was calculated with the Student's *t*-test. Thereafter, colonies were randomly selected and purified by streaking and re-streaking on fresh ZoBell 2216E agar media. Pure culture were transferred into ZoBell 2216E broth, and then incubated with shaking at 150 rpm until growth occurred. Subsequently, DNA from the each incubated culture was prepared by boiling a suspension of cells for 10 min, and amplified using PCR. The universal bacterial 16S rDNA primer pair 8F (5′-AGA GTT TGA TCA

  

TGG CTC AG-3′) and 1392R (5′-ACG GGC GGT GTG TRC-3′) (Amann, Ludwig & Schleifer 1995) was used for PCR amplification of 16S rDNA using a 2720 thermal cycler (Applied Biosystems, Foster city, CA, USA). The PCR reaction mixture (20 μL final volume) contained 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.05 U $\mu L^{-1}$ Taq PCRx DNA polymerase (Invitrogen, San Diego, CA, USA), 1× polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol $\mu L^{-1}$ of each primer. The PCR cycling was performed using following condition; 95°C for 5 min and then 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 90 s, before 7 min at 72°C. The reaction products were analysed by electrophoresis in 1.5% (w $v^{-1}$) agarose gel containing ethidium bromide (1 ng $mL^{-1}$).

### Extraction of bacterial genomic DNA and construction of DGGE fingerprinting technique of PCR amplicons

Bacterial genomic DNA from each homogenates was extracted using Promega DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's instruction. To obtain fingerprints of bacterial community in the different samples, bacterial 16S rDNA primer set 358F (5′-CCT ACG GGA GGC AGC AG-3′) with 40 bp GC clamp and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) (Romero & Navarrete 2006) was used to amplify 16S rDNA from total community DNA (100 ng) extracted for each sample by PCR. The PCR reaction mixture (50 μL final volume) contained 0.2 mM of dNTP, 0.05 U $\mu L^{-1}$ Taq PCRx DNA polymerase (Invitrogen), 1× polymerase reaction buffer, 2 mM $MgCl_2$, and 0.25 pmol $\mu L^{-1}$ of each primer. The PCR cycling was performed using following condition; 94°C for 5 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min, before 10 min at 72°C. The reaction products were analysed by electrophoresis in 1.5% (w $v^{-1}$) agarose gel containing ethidium bromide (1 ng $mL^{-1}$). The 16S rDNA amplicons were purified by the PCR preps DNA purification system (Promega) according to the manufacturer's instruction.

The PCR products were analysed by DGGE using a D-Code universal Mutation System (BioRad, Hercules, CA, USA) following the procedure described by Muyzer, De Waal and Uitterlinden (1993). The 16S rDNA amplicons (500 ng) were loaded onto 6% (v $v^{-1}$) polyacrylamide gel in 1× TAE with 30–50% gradient urea-formamide (100% corresponded to 7 M urea and 40% (v $v^{-1}$) formamide). Electrophoresis was conducted with a constant voltage of 20 V for 10 min and 200 V for 6 h in 1× TAE buffer at 60°C. The gel was stained in an ethidium bromide solution (0.5 μg $mL^{-1}$) in TAE buffer) and destained in distilled water for 20 min, before photographing using BioDoc-It® Imaging system (UVP, Upland, CA, USA). The DNA was obtained by elution of the excised bands into 20 μL of distilled water at 4°C overnight. An amount of 2 μL elute from individual bands was re-amplified and run on 1.5% (w $v^{-1}$) agarose gel containing ethidium bromide.

### Sequencing and biodiversity analysis

The PCR products and amplicons produced by re-amplification of the excised and eluted bands were purified and sequenced at an external laboratory (Macrogen, Seoul, South Korea) with the same primers used to produce them. The resulting chromatograms of DNA sequences were examined using Chromas 2.33. All sequences were examined for chimerism using a chimeric sequences detection program Bellerophon (Huber, Faulkner & Hugenholtz 2004). Homology searches were performed using sequences of more than 600 nucleotides for culturable bacteria (c. −400 nucleotides for amplicons from DGGE band) and close relatives were determined in GenBank databases using BLAST available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple alignments and calculation of distant matrixes were performed using MEGA version 4 (Tamura, Dudley, Nei & Kumar 2007). A phylogenetic tree was constructed using Neighbour-joining analysis function of MEGA version 4 with 1000 replicates in the bootstrap analysis. Distances were estimated with the Jukes–Cantor correction.

To analyse the bacterial diversity of culturable bacterial community, sequences with similarity of >97% were sorted as the same representative isolates group. Coverage values were calculated to determine how efficiently was described the complexity of the original bacterial community by the isolates. The coverage value is given as $C = 1 - (n_1 N^{-1})$, where $n_1$ is the number of isolates which occurred only once in the representative isolates group (Mullins, Britschgi, Krest & Giovannoni 1995). To investigate the bacterial diversity, Simpson's diversity index ($1 - \lambda$), evenness (J), species richness (d) and Shannon–Wiener Index ($H'$) were calculated using PRIMER6

software. The compositions of the DGGE band were compared using Sorensen similarity index, $Cs = 2j(a + b)^{-1}$, where $j$ is the number of DGGE band to both samples, and $a$ and $b$ are the numbers of DGGE band in A and B respectively.

### Nucleotide sequence accession numbers

The 16S rDNA sequences of the representative isolates and clones from obtained in this study have been deposited at DDBJ/EMBL/GenBank under accession no. AB822592 to AB822633.

## Results

### Morphological observation of different egg health condition

Morphological feature of a cluster of the fresh and infected egg were shown in Figure 1. Remarkable morphological difference was shown, although size was not different between the fresh and infected egg sample. Fresh egg sample was translucent to whitish colour and elliptical in shape (Fig. 1a), whereas infected egg sample had cream to brown colour and was elliptical in shape (Fig. 1b).

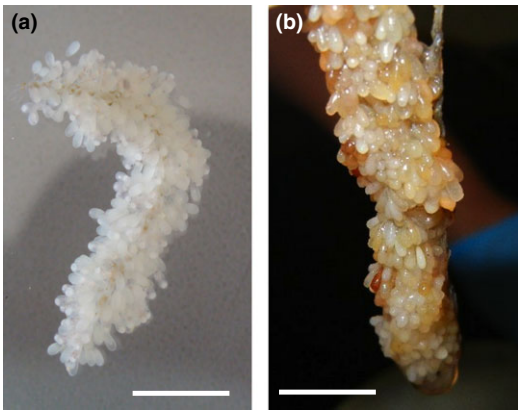


**Figure 1** Morphological observation of a cluster of octopus egg in different health condition. Fresh egg (a), and infected egg (b). White bar represent 1 cm.

### Enumeration and composition of culturable heterotrophic bacteria

There was a significant difference ($P < 0.05$) between the mean number of heterotrophic bacteria in the fresh egg and infected egg samples. The mean number of heterotrophic bacteria in the fresh egg was $5.35 \pm 0.104$ log (CFU $g^{-1}$), whereas in the infected egg sample was $7.15 \pm 0.0463$ log (CFU $g^{-1}$) (Table 1).

A total of 115 isolates from each sample were sequenced. Twenty-eight representative isolates groups were obtained at species level (>97% sequence identity) in comparison with sequences that had already deposited in the GenBank. All strains from the fresh egg sample were affiliated with lineages in the four domain Bacteria; α-, γ- and ε-proteobacteria and Bacteroidetes (Table 2). Bacterial community composition was substantially different between fresh and infected egg sample.

In the fresh egg sample, all isolates were phylogenetically affiliated with four divisions of the domain Bacteria; α-, γ- and ε-proteobacteria and Bacteroidetes. Predominant bacteria in the fresh egg were α- and γ-Proteobacteria. Dominant species was the genus *Rosebacter* (22 isolates). Second predominant species was the genus *Octadecabacter* counted nine isolates. Eight isolates belonged to genus *Amphritea*. The number of the *Vibrio* genus

**Table 1** Bacterial count and diversity of bacterial isolates from octopus egg samples

| | Bacterial count (log CFU $g^{-1}$)† | Number of isolates | Number of representative isolates group‡ | Coverage (C)§ | Evenness (J) | Richness (d) | Shannon index (H′) | Simpson's diversity index (1 − λ) |
|---|---|---|---|---|---|---|---|---|
| Fresh egg | 5.35 ± 0.104 | 59 | 20 | 0.780 | 0.841 | 4.660 | 2.520 | 0.907 |
| Infected egg | 7.15 ± 0.0463* | 56 | 8 | 0.946 | 0.754 | 1.739 | 1.569 | 0.751 |

*Indicates significant difference ($P < 0.05$; Student's $t$-test) between Fresh egg and infected egg values.
†Values are expressed as mean ± SE ($n = 3$).
‡Representative isolates group were categorized into 16S rDNA sequences of culturable bacterial strains with similarity of greater than 97%.
§Coverage (C) was calculated using the following formula $C = 1 - (n_1 N^{-1})$, where $n_1$ is the number of representative isolates group 545 with only one isolate and $N$ is the total number of isolates analysed (Mullins *et al.* 1995).

  

**Table 2** 16S rDNA sequences identified in the representative 546 isolates group from the octopus egg samples

| Sample | Phylotype | Accession number | No of isolates | Affiliation phylum/class | Closest sequence in database | Percentege identity |
|---|---|---|---|---|---|---|
| Infected egg | ODE1 | AB822592 | 17 | Proteobacteria/γ-proteobacteria | *Vibrio alginolyticus* (AF513447) | 99 |
| | ODE2 | AB822593 | 4 | Proteobacteria/γ-proteobacteria | *Pseudoalteromonas ruthenica* strain S3257 (FJ457210) | 100 |
| | ODE5 | AB822594 | 1 | Bacteroidetes/Flavobacteriia | *Tenacibaculum gallaicum* strain A37.1 (NR_042631) | 98 |
| | ODE7 | AB822595 | 8 | Bacteroidetes/Flavobacteriia | *Tenacibaculum mesophilum* strain MBIC1140 (NR_024736) | 98 |
| | ODE8 | AB822596 | 21 | Proteobacteria/γ-proteobacteria | *Pseudoalteromonas* sp. S1941 (FJ457166) | 100 |
| | ODE22 | AB822597 | 1 | Proteobacteria/γ-proteobacteria | *Pseudoalteromonas mariniglutinosa* strain: Do-80 (AB257337) | 99 |
| | ODE28 | AB822598 | 3 | Proteobacteria/γ-proteobacteria | *Vibrio splendidus* strain GHrC13 (GQ375456) | 99 |
| | ODE68 | AB822599 | 1 | Proteobacteria/γ-proteobacteria | *Pseudoalteromonas piscicida* strain 1314 (GU726846) | 100 |
| Fresh egg | OFEM1 | AB822600 | 8 | Proteobacteria/γ-proteobacteria | *Amphritea atlantica* strain M41 (NR042455) | 97 |
| | OFEM2 | AB822601 | 9 | Proteobacteria/α-proteobacteria | *Octadecabacter* sp. UDC483 (HM032014) | 97 |
| | OFEM3 | AB822602 | 1 | Proteobacteria/α-proteobacteria | *Roseobacter* sp. D4024 (FJ161256) | 98 |
| | OFEM4 | AB822603 | 4 | Proteobacteria/γ-proteobacteria | *Vibrio communis* strain CAIM 1308 (HM584082) | 99 |
| | OFEM7 | AB822604 | 2 | Proteobacteria/α-proteobacteria | *Roseobacter* sp. RED1 (AY136122) | 99 |
| | OFEM8 | AB822605 | 8 | Proteobacteria/α-proteobacteria | *Roseobacter* sp. 14III/A01/004 (AY576690) | 100 |
| | OFEM9 | AB822606 | 11 | Proteobacteria/α-proteobacteria | *Roseobacter* sp. 38.98 (AY870684) | 99 |
| | OFEM13 | AB822607 | 1 | Proteobacteria/γ-proteobacteria | *Halomonas pacifica* strain H1704 (JF346669) | 97 |
| | OFEM14 | AB822608 | 1 | Proteobacteria/α-proteobacteria | *Phaeobacter* sp. UDC452 (HM032005) | 99 |
| | OFEM17 | AB822609 | 1 | Bacteroidetes/Cytophagia | *Flexibacter aurantiacus* subsp. *copepodarum* strainIFO 15978 (AB078044) | 98 |
| | OFEM18 | AB822610 | 4 | Proteobacteria/α-proteobacteria | *Ruegeria scottomollicae* strain prru1 (FN821687) | 99 |
| | OFEM31 | AB822611 | 1 | Proteobacteria/α-proteobacteria | *Roseovarius aestuarii* strain USC61 (HQ441227) | 100 |
| | OFEM44 | AB822612 | 1 | Proteobacteria/γ-proteobacteria | *Vadicella arenosi* strain: KMM 9008 (AB564597) | 96 |
| | OFEM51 | AB822613 | 1 | Bacteroidetes/Flavobacteriia | *Tenacibaculum* sp. MOLA 533 (AM990757) | 100 |
| | OFEM56 | AB822614 | 1 | Proteobacteria/ε-proteobacteria | *Arcobacter* sp. MA5 (AB542077) | 100 |
| | OFEM60 | AB822615 | 1 | Proteobacteria/γ-proteobacteria | *Colwellia* sp. STAB 604 (JF825446) | 98 |
| | OFEM62 | AB822616 | 1 | Proteobacteria/α-proteobacteria | *Roseovarius pelophilus* strain HK7 (EU939692) | 98 |
| | OFEM63 | AB822617 | 1 | Proteobacteria/α-proteobacteria | *Jannaschia helgolandensis* strain M21551 (HM032794) | 98 |
| | OFEM64 | AB822618 | 1 | Proteobacteria/α-proteobacteria | *Phaeobacter* sp. LSS9 (GQ906799) | 97 |
| | OFEM66 | AB822619 | 1 | Proteobacteria/α-proteobacteria | *Litoreibacter* sp. MA1-1 (JN021667) | 98 |

**Table 3** Representative denaturing gradient gel electrophoresis (DGGE) band sequences 549 from the octopus egg samples

| DGGE band | Base pair | Accession number | Affiliation phylum/class | Closest relative | Similarity (%) | Fresh egg | Infected egg |
|---|---|---|---|---|---|---|---|
| E1 | 398 | AB822620 | Bacteroidetes/Cytophagia | *Flexibacter* sp. S4471 (FJ457296) | 96 | • | |
| E2 | 405 | AB822621 | Bacteroidetes/Flavobacteriia | Uncultured Flavobacteriaceae bacterium clone S.o-5 (HM031429) | 99 | • | • |
| E3 | 390 | AB822622 | Bacteroidetes | Uncultured Bacteroidetes bacterium clone Jc_OTU22 (HM593572) | 95 | • | • |
| E4 | 416 | AB822623 | Bacteroidetes/Cytophagia | *Flexibacter* sp. UST991130-045 (AF465362) | 99 | • | • |
| E5 | 406 | AB822624 | Unidentified | Uncultured bacterium clone PEACE2006/124_P3 (EU394641) | 93 | | • |
| E6 | 423 | AB822625 | Unidentified | Uncultured organism clone ctg_CGOCA51 (DQ395549) | 98 | | • |
| E7 | 400 | AB822626 | Bacteroidetes | Bacteroidetes bacterium S10/1 (AY847473) | 100 | | • |
| E8 | 380 | AB822627 | Bacteroidetes/Cytophagia | Uncultured *Flexibacter* sp. clone ZS-4-382 (FN668197) | 97 | | • |
| E9 | 400 | AB822628 | Proteobacteria/α-proteobacteria | *Roseovarius* sp. MA1-10 (HQ852039) | 99 | • | |
| E10 | 400 | AB822629 | Proteobacteria/α-proteobacteria | *Sulfitobacter* sp. KMUT3 (AB583769) | 98 | • | |
| E11 | 392 | AB822630 | Proteobacteria/α-proteobacteria | *Ruegeria* sp. Rg351 (HE818387) | 98 | • | |
| E12 | 389 | AB822631 | Proteobacteria/α-proteobacteria | *Phaeobacter* sp. Ph82 (HE818248) | 98 | • | |
| E13 | 402 | AB822632 | Proteobacteria/α-proteobacteria | *Roseobacter* sp. WED1.1 (AY536562) | 99 | • | |
| E14 | 402 | AB822633 | Proteobacteria/α-proteobacteria | Uncultured Rhodobacteraceae bacterium clone GG101008Clone72 (JN591912y) | 99 | | • |

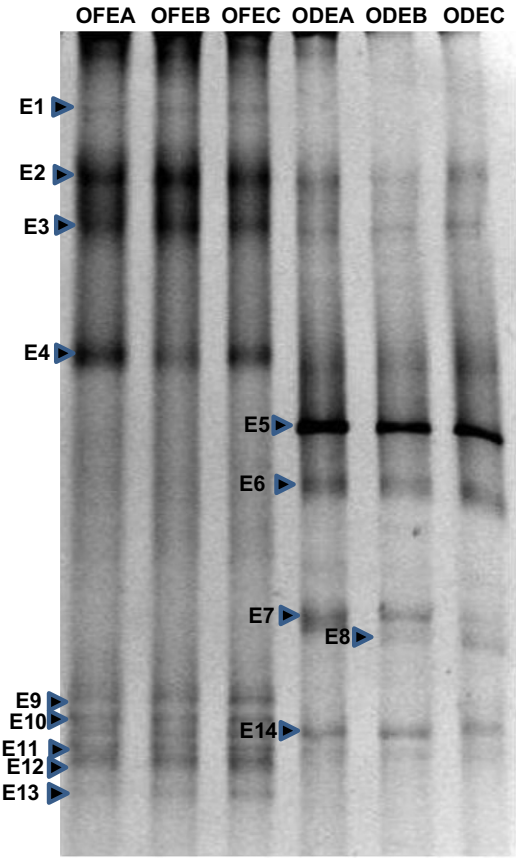


**Figure 2** Denaturing gradient gel electrophoresis band profile of microbiota in fresh and infected octopus egg. Each lane shows the bacterial composition of one individual. OFE and ODE mean octopus fresh egg and octopus infected egg samples respectively. A, B and C represent triplicates. The triangles show the sequenced bands.

and the *Ruegeria* genus was four isolates respectively. Two strains from each genus, *Phaeobacter* and *Roseovarius*, were isolated respectively. There were many unique strain isolated from the fresh egg sample (Table 2); the genus *Halomonas*, *Flexibacter*, *Vadicella*, *Tenacibaculum*, *Arcobacter*, *Colwellia*, *Jannashia* and *Litoreibacter*.

In contrast to the fresh egg sample, the predominant bacteria in the infected egg sample were γ-proteobacteria. All strains from the infected egg sample were affiliated with lineages in the two domain Bacteria; γ-proteobacteria and Bacteroidetes. Predominant species was the genus *Pseudoalteromonas* (27 isolates) and second predominant species was *Vibrio* (20 isolates). Nine isolates belonged to *Tenacibaculum*.

Diversity index of the culturable bacterial community in the octopus egg samples was presented


 

**Table 4** Comparison of the diversity 551 of bacterial isolates from octopus egg samples

| Sorensen similarity index (%) of each octopus egg sample | | | | | |
|---|---|---|---|---|---|
| | **OFEA** | **OFEB** | **OFEC** | **ODEA** | **ODEB** |
| OFEB | 100 | | | | |
| OFEC | 100 | 100 | | | |
| ODEA | 35.3 | 35.3 | 35.3 | | |
| ODEB | 30.0 | 30.0 | 30.0 | 80.0 | |
| ODEC | 31.6 | 31.6 | 31.6 | 71.4 | 82.4 |

OFE and ODE mean octopus fresh egg and octopus infected egg samples, respectively. A, B and C represent triplicates.

in Table 1. Coverage of bacterial diversity of fresh and infected egg samples was 78.0% and 94.6% respectively. The whole diversity index of the fresh egg sample was much higher than that of the infected egg sample (Table 1).

### DGGE fingerprinting technique

To compare the structure of the bacterial community of octopus egg samples in different health condition, PCR-DGGE fingerprinting technique was developed. Figure 2 shows the DGGE profiles of PCR amplified 16S rDNA obtained from DNA extracted directly from each egg sample. Selected dominant and intense bands were subsequently sequenced to describe the phylogenetic diversity of the −400 bp partial 16S rDNA sequence (Table 3).

The triplicated DGGE band profile that demonstrated the bacterial diversity of fresh and infected egg sample was shown in Figure 2. Total band number of fresh egg sample was similar to that of the infected ones, however DGGE banding patterns differed substantially between fresh and infected egg sample. The sequences of DGGE bands were affiliated with Bacteroidetes and α-proteobacterium. The bacterial community of fresh egg sample (E1-E4 and E9-E13) was composed of Bacteroidetes and α-proteobacterium, whereas Bacteroidetes was predominant in the infected egg sample (E2-E8 and E14) (Table 3). Bands E1, E4 and E8 were closely related to the genus *Flexibacter*, which showed similarities of 96%, 99% and 97% respectively. Bands E2, E9, E13 and E14 showed 99% similarity to the genus uncultured Flavobacteriaceae bacterium clone S.o-5, *Roseovarius*, *Roseobacter* and uncultured Rhodobacteraceae bacterium clone GG101008Clone72 respectively. Bands E6, E10, E11 and E12 showed 98% similarity to the uncultured marine bacterium clone KG_A3_120 m182, genus *Sulfitobacter*, *Ruegeria* and *Phaeobacter* respectively. Band E7 showed 100% similarity to the Bacteroidetes bacterium S10/1. Band E3 showed 95% similarity to the uncultured Bacteroidetes bacterium clone. Band E5 was related to the uncultured bacterium clone PEACE2006/124_P3 (93% of similarity).

There was high similarity in bacterial composition among the triplicates of fresh and infected egg sample (Fig. 2; Table 4). The highest Cs value was 100% among fresh egg samples (OFEA, OFEB and OFEC) and the lowest Cs value was 30.0% between fresh egg group (OFEA, OFEB and OFEC) and infected egg (ODEB). The Cs value of the fresh egg group was 100% and that of the infected egg group ranged from 71.4% to 82.4% (Table 4).

## Discussion

Poor egg quality and resulting in mass mortalities have been serious problems in larval production systems. Moreover, the microflora on eggs and in larval incubators may affect the short- and long-term health of farmed fish (Olafsen 2001). Octopus egg mortality occurred was mainly due to the detaching from egg cluster or attributed to contaminated egg when were removed by the female (González *et al.* 2008). Until now, some researchers reported that fish egg associated bacteria had a potential of causing mortality in marine fish eggs (Nelson & Ghiorse 1999; Verner-Jeffreys, Nakamura & Shields 2006; McIntosh, Ji, Forward, Puvanendran, Boyce & Ritchie 2008). In the case of octopus, Uriarte *et al.* (2011) suggested that *Enteroctopus megalocyathus* egg was susceptible to microbial infections adhered to their bodies and resulting in death. Thus, microbiological monitoring becomes important in the egg incubation and paralarvae stage. However, information on the microbiological monitoring of octopus egg is still scarce. In the present study, we investigated and compared the bacterial community composition of *Octopus mimus* egg in different health condition using culturable and unculturable methods.

Total heterotrophic bacterial number in the infected egg sample was significantly higher than the fresh egg sample in the present study. This is in accordance with other reports. Mickėnienė and Šyvokienė (2011) showed that the abundance of

total heterotrophic and pigmented bacteria on dead egg of noble crayfish (*Astacus astacus L.*) was much higher than on fresh egg. Hameed (1997) suggested that dead eggs might release some nutrients into the culture water, nourishing the bacteria which have already attached to the fresh eggs. These results suggest that the significant increase in the total heterotrophic bacterial number in the infected octopus egg in our study is caused by both already attached to the egg bacteria and free-living bacteria easily colonize on the infected egg surface and proliferate using the released nutrition from infected egg.

In the present study, unculturable bacterial community of the infected egg sample was significantly different from culturable bacterial community and most of them showed high similarity to the unculturable bacterium clones. It is speculated that most of bacteria from the infected egg sample did not have colonization activity. This is supported by the study of Koren and Eugene (2006), which indicated that 99.8% bacteria in the coral mucus and tissue samples failed to produce colonies on Marine agar. Furthermore, culturable bacterial diversity in the infected egg sample was substantially decreased as compared to the fresh egg sample in contrast to total heterotrophic bacterial number, and the composition of culturable and unculturable bacterial community was also dramatically changed in accordance with their health condition. This result is opposed to the case of coral, in which the diversity of bacterial species is higher in diseased corals (Reis, Araújo, Moura, Francini-Filho, Pappas, Coelho, Krüger & Thompson 2009; Sunagawa, Todd, Yvette, Eoin, Michael, Christian, Ernesto, Gary & Monica 2009). This is because *Octopus mimus* female carries their eggs by their arms and secrete mucus to protect them; in consequence it is assumed that some bacteria could colonize the surface of the infected egg easier than the fresh egg due to detachment from their arms and loss of mucus protection. Until now, there is some information on the relationship of surface mucus and microbiota (Bernadsky & Rosenberg 1992; Koren & Eugene 2006; Staroscik & Nelson 2008). The surface mucus of many marine eukaryotes is covered by microorganisms that play an important role in the life of the host organism (Penesyan, Marshall, Holmstrom, Kjelleberg & Egan 2009). Guo, Huang, Huang, Zhao and Ke (2009) demonstrated that pedal mucus and the mucus trail of the small abalone *Haliotis diversicolor* improved bacterial growth, attachment and biofilm formation, and also altered bacterial community structure. These studies indicated that mucus component and bacteria on the mucus might construct some important relationship, and a better understanding of bacterial community on the mucus might be valuable to identify potential useful bacteria as probiotics in the octopus aquaculture. Therefore, we need further investigation to evaluate the difference in the bacterial community composition between egg and female mucus, and the effect of mucus component on the bacterial community.

In the present study, the proportion of *Roseobacter* clade in the fresh egg sample was much higher than that of the infected egg sample. *Roseobacter* clade are from various ecological niches and phylogenetically diverse, and more demonstrate enormous interest in the genetic and metabolic diversity (Brinkhoff, Helge-Ansgar & Meinhard 2008). Some researchers reported that this clade bacterium was detected in squid (Grigioni, Boucher-Rodoni, Demarta, Tonolla & Peduzzi 2000; Barbieri, Bruce, Deborah, Ludek, Duane, Andreas & Mitchell 2001; Pichon, Valeria, Mark & Renata 2005), however no information on its isolation or detection from octopus has been reported in recent articles. In the present study, we reported the isolation and detection of *Roseobacter* clade from octopus for the first time. Moreover, there are some reports of antibiotic production by bacteria belonging to the *Roseobacter* clade and utilization as a probiotics (Planas, Pérez-Lorenzo, Hjelm, Gram, Fiksdal, Bergh & Pintado 2006; Kesarcodi-Watson, Heinrich, Josie & Lewis 2008; Nissimov, Eugene & Colin 2009; D'Alvise, Jette, Cisse, Kristian & Gram 2010; Gram, Jette & Jesper 2010; Sharifah & Eguchi 2011; D'Alvise, Siril, Heidrun, Gram & Bergh 2013). These results raise the possibility that *Roseobacter* clade, which was isolated in the present study, is also correlated with the octopus egg health condition and may be utilized as probiotics to control bacterial community and improve the growth parameter of egg. Therefore, we need further investigation to elucidate whether these isolates can use as probiotics for octopus egg culture. Moreover, several authors reported that Bacteroidetes was detected in squid and other octopus species, although it remains unclear that these bacteria are associated with host health condition (Pichon *et al.* 2005; De la Cruz-Leyva, Zamudio-Maya, Corona-Cruz, González-De la Cruz

& Rojas-Herrera 2011). In addition, De Castro, Samuel, Alessandra, Rodrigo, Ronaldo, Georgios, Thiago, Thompson and Ricardo (2010) suggested that Bacteroidetes played a role in coral health, since the rate of Bacteroidetes was significantly increasing in the disease coral, although Bacteroidetes was presented in both health conditions. In the present study, Bacteroidetes was presented in both health conditions, however the proportion of these bacteria was higher in the infected egg sample of both culturable and unculturable bacterial community, which is in contrast to *Roseobacter* clade. Our result of culturable and unculturable methods shows that *Roseobacter* clade and Bacteroidetes were detected in both health conditions although their proportion was different, assuming that not only *Roseobacter* clade but also Bacteroidetes might play some role in octopus egg health condition.

On the other hand, the genus *Pseudoalteromonas* and *Vibrio* were predominant within the culturable bacterial community associated with the infected egg, although not detected in the DGGE gel. This is probably because these bacteria were better cultured using ZoBell 2216E agar. Several *Pseudoaltoromonas* spp. was known as one of the fish pathogen (Nelson & Ghiorse 1999; Pujalte, Ariadna, María, Pilar & Esperanza 2007; Sandaa, Laila, Thorolf & Øivind 2008). Moreover, *Pseudoalteromonas* spp. has been detected in infected octopus egg from *Enteroctopus megalocyathus* (Uriarte *et al.* 2011). In the present study, there was about 50% of the genus *Pseudoalteromonas* from the culturable bacterial community in the infected egg sample in spite of no detection from the fresh egg sample. *Vibrio* spp. are natural habitants of seawater and broadly distributed throughout the world. Among those, *Vibrio alginolyticus* and *V. splendidus* have been considered pathogenic strains of the aquatic animals (Gatesoupe, Christophe & Nicolas 1999; Liu, Ji-Yang, Pei-Tze & Kuo-Kau 2004; Jayaprakash, Pai, Philip & Singh 2006; Garnier, Yannick, Celine, Maeva & Nicolas 2007; Xu, Dan-Li, Chao-Yan, Shan, Chun-Lin & Xiu 2013). Proportion of the genus *Vibrio* in the infected egg sample was significantly higher than the fresh egg sample in the present study. Moreover, *Vibrio* spp. known as fish pathogen (*Vibrio alginolyticus* and *Vibrio splendidus*) was isolated only from the infected egg sample. These results speculate that these bacteria raise the possibility of pathogenic bacteria to *Octopus mimus* egg.

To our knowledge, this is the first study to demonstrate the bacterial community associated with the egg of Chilean octopus *Octopus mimus* and to reveal that this bacterial community might be influenced by their health condition. Our results suggest that some bacteria such as *Roseobacter* clade and Bacteroidetes might be correlated with the octopus egg health condition. Furthermore, it raises the possibility that some γ-proteobacteria might be the pathogenic bacteria candidate to octopus egg. Therefore, the results presented in this study are beneficial for the future of octopus aquaculture, although we need to do further bioassays to understand better the disease problems associated with the octopus egg. Furthermore, it is important to reveal the change in bacteria community under different growth stage (e.g. egg, paralarvae and juvenile) to obtain more detail information on the bacterial community, their dynamics and relationship with host animal.

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