Aquaculture 338-341 (2012) 147–153

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Changes in gut-associated flora and bacterial digestive enzymes during the development stages of abalone (*Haliotis diversicolor*)

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ARTICLE INFO

Article history:
Received 12 October 2011
Received in revised form 14 January 2012
Accepted 17 January 2012
Available online 25 January 2012

Keywords:
Haliotis diversicolor
Development stage
Gut bacterium
Digestive enzyme
Diet

ABSTRACT

The gut of abalone contains a diverse population of bacteria which contribute to food digestion and nutrient absorption. To investigate the development of gut microflora in the small abalone *Haliotis diversicolor* and the effect of various diets on the bacterial communities, we analyzed the composition of gut microbial flora and the bacterial digestive enzymes in connection with four important development stages, i.e., larva, early-juvenile, late-juvenile and grow-out. Among these cultivated bacteria, the Firmicutes group was isolated from both the larval and early-juvenile stages; the CFB (*Cytophaga/Flexibacteria/Bacterioides*) group was detected in early juveniles, late juveniles and grow-out; the γ-proteobacteria existed throughout the four development stages. The isolated *Bacillus* sp. and *Pseudoalteromonas* sp. dominated in the larvae and were rarely detected in other stages. Subsequently, the genus *Vibrio* predominated in the latter three phases after the first feeding. Cluster analysis of the DGGE (Denaturing Gradient Gel Electrophoresis) patterns was basically congruent with the result of culture-dependent method in which the gut bacterial community indicated the successional change in the development stages. And the artificial diets contribute to the change of gut bacterial composition to some extent. The enzyme assay showed that the gut bacteria isolated from the early juveniles produced the greatest diverse digestive enzymes compared with those isolated from other development stages. Most cultivated bacteria in each development stage showed protease, lipase or alginate-degrading activities which could help the farmed abalone *H. diversicolor* to digest diets. All results showed that a unique but interrelated bacterial flora was developed in the abalone gut which was coincident with the change of diets.

1. Introduction

China is one of the key abalone producing countries in the world, with 5000 and 3000 tons per annum from the mainland and Taiwan, respectively (Roberto et al., 2007). Small abalone *Haliotis diversicolor* is naturally distributed along the coastal waters of East Asia from Japan to the Philippines, and it is a commercially important species cultured along the coast of southern China (Cai and Wang, 2008). According to the diets style, the growth process of the farmed *H. diversicolor* was divided into four development stages, including larva without any food, early-juvenile with diatom, late-juvenile with artificial food and grow-out with red seaweed. The abalone is a grazer that can introduce diverse microbial flora into the gastrointestinal tract through diets.

The structure and composition of the gut flora are the reflection of natural selection of the microbial and host levels, which promotes mutual cooperation within and functional stability of this complex ecosystem (Harris, 1993; O'Haral and Shanahan, 2006). Some studies have showed that microbial flora existing in the gut of abalone probably played important roles. Such roles include algal polysaccharide degradation, maintenance of pH and redox potential in the intestinal as well as preventing foreign microbes from invading (Tanaka et al., 2003, 2004). Because of the vital role of the intestine bacterial flora for the host's survival, it could be assumed that the most important microbe groups are always present regardless of selective pressure conditions (Husseneder et al., 2009). Erasmus et al. (1997) suggested that predominant resident bacteria in the digestive system of *Haliotis midae* possessed algal polysaccharide-degrading ability. Both Tanaka and Sawabe also showed that a unique alginate-degrading bacterium *Vibrio* sp. was the dominant species in abalone *Haliotis discus hannai* (Sawabe et al., 1998; Tanaka et al., 2003). In the previous study, we isolated the genera *Vibrio*, *Flammeovirga*, *Shewanella* and *Persicobacter* which were isolated from the adult abalone *H. diversicolor* (Huang et al., 2010). However, in the development stage of abalone, how gut microflora develop, and whether the gut microflora change with shift in diet remain unclear in the farmed abalone *H. diversicolor*.

With extremely diverse of species, the metabolic capacity of gut microflora can show positive and negative effects on host (Macfarlane and

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doi:10.1016/j.aquaculture.2012.01.016

Cummings, 1991; Rastall, 2004). Since late 2000, *H. diversicolor* in China has experienced mass mortality with diverse symptom and pathogens in late-juvenile and grow-out stages (Cheng et al., 2004). The combined loss of nutrients from impaired absorption in the intestine, as well as a loss of digestive enzyme production, could result in disease followed by death (Friedman et al., 2000). Microflora balance has a profound impact on the host health and disease through their function in the development and maintenance of host physiology, immunology and also host nutrition (Blaut and Clavel, 2007; Cash, et al., 2006; Marchesi and Shanahan, 2007). Therefore, it was speculated that the susceptibility of small abalone to disease may due to negative interactions between gut associated flora and host organism. For instance, controlling intestinal microbial balance of abalone may potentially influence the absorption of nutrition and stimulation of host immune responses (Gómez and Balcázar, 2008). To date, the variety of bacterial digestive enzymes in abalone *H. diversicolor*, which was fed on varied diets in the development process, were less pronounced by the researchers yet.

Here we describe the variability and succession of intestinal microflora and bacterial digestive enzyme with the change of diets during the *H. diversicolor* growth process. This information could help to understand the correlation between intestinal microflora and the farmed abalone, and further control the gut microbial communities.

2. Materials and methods

2.1. Abalone rearing conditions and collection

The growth of small abalone *H. diversicolor* was arbitrarily divided into four stages according to the different dietary style, larva, early-juvenile, late-juvenile and grow-out. From fertilization to larva, the abalone did not take any food and is wholly reliant on endogenous reserves. After four days, the larva began the process of settlement and metamorphosis, and then transferred into early juveniles which fed the benthic diatoms on the plastic film. The mix benthic diatoms were added into the culture pool to form the biofilm, including *Achnanthes javanica* var., *Amphora fluninensis*, *Cocconeis diminuta* and *Nitzschia alexandrina* (Guo et al., 2007). After peeling off from the diatom substrate, abalones transferred into late-juvenile stage, and the artificial diets (provided by Zhangzhou Changshan President Lianfengxingye Forage Co. Ltd) were added under foursquare cement brick for 3–4 months. The artificial diet is composed of fish powder, seaweed powder, soybean powder, vitamins, flour, minerals etc. The physical properties were presented in Supplementary Table A1. Thereafter, the late-juvenile was transported into an abalone-cage for field grow-out. The red algae *Gracilaria* sp. was mainly fed as food for about 1 year by the way of flowing with natural seawater. Immediately after harvest of *Gracilaria* sp., the algal biomass was rinsed using sand filtered and ozone-treated water and subsequently pre-cultured in an aerated container before feeding. Every two days, the residual algae and excrement were siphoned off and fresh algae were re-supplied again. Abalone samples were obtained from a commercial abalone farm (Zhang zhou, Fujian, China) in different mariculture phases. All abalones were derived from the same spawn and each experiment was composed of three replicates. The different sizes of abalone raised under different culture conditions are shown in Table 1.

2.2. Sample preparation and isolation of the cultivable bacteria

All samples were taken into the laboratory within 2 h in an ice-box. As the small size rendered dissection impractical, larval and early-juvenile abalone were used as integer to sample the aerobic bacterial flora in the following experiments. The shells of early-juveniles were cut away before treatment. To remove the surface bacteria, all samples were disinfected in a 0.1% (w/v) benzalkoniumchloride solution for 15 s (Huys et al., 2001), and then rinsed three times in a sterile natural seawater, each rinse for 5–10 s. Next, the larvae, early-juveniles and the intestine extending from the stomach to the anus which had been aseptically excised from small abalone with sterile forceps, were prepared the homogenate previously described (Sawabe et al., 1998). Subsequently, all samples were randomly divided into two groups, and each group was placed into a 15 ml tube. One pooled sample was used for bacterial cultivation. Another was used for DGGE analysis, in which all samples were washed twice with sterile sodium-phosphate buffer, pH 7.4, and stored at −20 °C until total DNA extraction.

Serial 10-fold dilutions (10^{-2} to 10^{-4}) of the homogenized sample solutions were prepared using sterile natural seawater and 0.1 ml volumes were plated on Marine Agar 2216E (MA). All plates were prepared for triplicates and incubated at 22 °C for 5 days. The distinct colony phenotypes (size, pigmentation, opacity, texture, form, elevation, margin, or surface) both on MA, were picked off, then streaked onto 2216E solid media and grown at 28 °C overnight finally. The pure cultures were then preserved at −70 °C containing 20% (V/V) glycerol. The isolates obtained from different development stages were designated as HL (larva), HE (early juvenile), HJ (late juvenile) and HG (grow-out).

2.3. 16S rRNA sequence identification of bacterial strains isolated from abalone

The genomic DNA of the pure bacterial cultures was subjected to sodium dodecyl sulfate (SDS)/phenol-chloroform-isoamyl alcohol extraction methods (Sambrook et al., 1989). The universal primers 27F and 1492R were used to amplify the 16S rRNA full-length gene of the cultivable bacteria (DeLong, 1992). Amplified 16S rRNA gene sequences (~1500 bp) of pure strains were subjected to RFLP analysis using enzymes *Rsa*I (GT′AC) and *Msp*I (C′CGG) to identify distinct strains. The strains with different digestion profiles were picked and ligated into the pMD19-Tclone (TaKaRa Biotechnology). The sequences cloned were further sequenced (Sangon Biotechn Co., Ltd). The 16S rRNA sequences determined were checked for similarities by using BLAST in NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST/). All 16S rRNA sequences of bacterial isolates presented in this study have been deposited in the EMBL database under the accession numbers of FN554585–FN554617 (Table 2).

2.4. Screening for digestive enzyme from bacteria isolates

Six main digestive related enzymes (agarase, amylase, protease, lipase, cellulase and alginate-degrading enzyme) were screened out from all isolates to investigate the relationship between the gut bacterial communities and their diets. Pure cultures were inoculated into medium

Table 1
Abalone samples used in this study to determine the gut bacterial flora of *H. diversicolor*.

Sample stage	Age of sample	Diet	No. of abalones	Average body length of abalone individuals (mm)	Viable bacterial counts (CFU/g)	No. of bacterial species
Larva (HL)	4 days	No food	100 μl	0.3 ± 0.1	3.3×10^4	5
Early juvenile (HE)	40 days	Diatoms	100 μl	3 ± 0.4	2.1×10^5	11
Late juvenile (HJ)	150 days	Artificial diets	5	20 ± 2.1	6.8×10^5	7
Grow-out (HG)	~1 year	*Gracilaria* sp.	5	59 ± 3.0	4.5×10^6	10

Table 2
Cultured bacteria associated with the abalone gut in four development stages.

Sample stage	No. of isolates	Accession number	Affiliation	Closest sequence in database	Identity (%)
Larvae	HL1	FN554585	Gram-positive bacteria	*Bacillus* sp. NH6 (EU107757)	99
	HL2	FN554586	Gram-positive bacteria	*Bacillus* sp. HNL14 (EU373352.1)	99
	HL3	FN554587	Gram-positive bacteria	*Bacillus pumilus* (AB308441.1)	99
	HL4	FN554588	γ-Proteobacteria	*Pseudoalteromonas* sp. JL1003 (DQ985032.1)	99
	HL5	FN554589	γ-Proteobacteria	*Pseudoalteromonas* sp. S9 (U80834.1)	99
early-juvenile	HE1	FN554590	γ-Proteobacteria	*Vibrio alginolyticus* DH51 (FJ404753.1)	98
	HE2	FN554591	γ-Proteobacteria	*Vibrio harveyi* (AY911396.1)	99
	HE3	FN554592	γ-Proteobacteria	*Vibrio* sp. (FM957470.1)	99
	HE4	FN554593	Gram-positive bacteria	*Bacillus* sp. A10 ZZ-2208 (FM180514.1)	99
	HE5	FN554594	Gram-positive bacteria	*Bacillus megaterium* (EU880506.1)	99
	HE6	FN554595	CFB group	Uncultured *Flavobacteriales bacterium* (FJ403106.1)	98
	HE7	FN554596	γ-Proteobacteria	*Vibrio* sp. MY-2008-U34d (FM957474.1)	99
	HE8	FN554597	γ-Proteobacteria	*Vibrio* sp. BWDY-62 (DQ328947.1)	99
	HE9	FN554598	γ-Proteobacteria	*Vibrio* shilonii (AY911395.1)	99
	HE10	FN554599	γ-Proteobacteria	Mucus bacterium 49 (AY654779.1)	99
	HE11	FN554600	γ-Proteobacteria	*Vibrio harveyi* (AY911396.1)	97
late-juvenile	HJ1	FN554601	γ-Proteobacteria	*Vibrio* sp. LMG 23856 (EF599163.1)	99
	HJ2	FN554602	γ-Proteobacteria	*Vibrio* sp. V004 (DQ146977.1)	98
	HJ3	FN554603	γ-Proteobacteria	*Shewanella* sp. IRI-160 (AY566557.1)	99
	HJ4	FN554604	γ-Proteobacteria	*Vibrio tapetis* (AY129278)	99
	HJ5	FN554605	CFB group	*Aquimarina latercula* (M58769.2)	98
	HJ6	FN554606	γ-Proteobacteria	*Agarivorans* sp. JAM-A1m (AB426125.1)	98
	HJ7	FN554607	γ-Proteobacteria	*Vibrio shilonii* (AY911395.1)	99
Grow-out	HG1	FN554608	γ-Proteobacteria	*Photobacterium* sp. 04104 (AM422803.1)	99
	HG2	FN554609	γ-Proteobacteria	*Shewanella waksmanii* (AY170366.1)	99
	HG3	FN554610	γ-Proteobacteria	*Vibrio shilonii* (AY911395.1)	99
	HG4	FN554611	Sphingobacteria	*Flammeovirga aprica* (AB247553.1)	98
	HG5	FN554612	γ-Proteobacteria	*Vibrio harveyi* (FJ605242.1)	99
	HG6	FN554613	γ-Proteobacteria	*Photobacterium* sp. FL3D2 (DQ317673.1)	96
	HG7	FN554614	γ-Proteobacteria	*Vibrio harveyi* LA08005 (GQ180186)	99
	HG8	FN554615	γ-Proteobacteria	*Vibrio harveyi* XC08001 (FJ605242.1)	99
	HG9	FN554616	CFB group	*Tenacibaculum* sp. PS4-6 (EU651805.1)	97
	HG10	FN554617	γ-Proteobacteria	*Shewanella aquimarina* (FJ589034.1)	99

containing varied substrates, and some isolates with digestive enzyme activities were screened. The following is the screening media components: 1) amylase activities: yeast extract 0.5% (w/v), soluble starch 1.5%(w/v), agar 1.5% (w/v); 2) protease activities: de-fatted milk powder 1% (w/v), tryptone 1% (w/v), agar 1.5% (w/v); 3) lipase activities: yeast extract 0.5% (w/v), tryptone 1% (w/v), tributyrin 1% (w/v), agar 1.5% (w/v); 4) cellulase activities: yeast extract 0.5% (w/v), CM-cellulose, sodium salt 0.5% agar 1.5% (w/v); 5) alginate-degrading enzymatic activities: sodium alginate 0.5% (w/v), agar 1. 5% (w/v). All medium were dissolved in seawater, and prepared standardly (pH 7.4). Finally, all plates were prepared in three replicates and incubated at 28 °C overnight. Different colonies producing transparent circles and/or the sunken colony types, represented strains with digestive enzyme activities, were selected for further analysis.

2.5. Comparison of the bacterial composition using DGGE

Samples including diets, seawater in mari-farm, larval and early-juvenile body and the intestine of abalone were collected for further analysis. The dietary samples (diatoms, artificial food or red algae *Gracilaria* sp.) were collected immediately prior to the next feeding respectively, at this point the abalone were considered to have a minimum quantity of undigested food remaining from their previous meal and hold a stable microflora associated with this stage abalone. The DNA from all samples was extracted following the protocol described by Harder et al. (2003). The purity and molecular size of the extracted DNA were estimated by agarose gel electrophoresis. Variable regions V of bacterial 16S rDNA sequences were amplified by touch-down PCR using the primer set 341F (5′-CCTACG GGAGGCAGCAG-3′) and 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′) with GC clamps attached to the forward primer (Muyzer et al., 1993). PCR products were run on a 10% acrylamide gel with 30–55% linear gradient of urea and formamide, and electrophoresed at 100 V and 60 °C for 10 h in 1 × TAE buffer. Then, the gel was stained with silver dye. DGGE band position and intensity were determined using Cross Checker software (http://www.dpw.wau.nl/pv/pub/crosscheck/), and they were carefully modified manually. DGGE band position and intensity were determined with 1.00% position tolerance and 1.00% optimization. Cluster analysis was performed based on the Pearson similarity correlation and Ward dendrogramming method. A dendrogram was obtained using the unweighted pair-group clustering method with arithmetic averages (UPGMA).

3. Results

3.1. Quantification and molecular identification of cultivated intestinal bacteria

The bacterial counts of abalone at four development stages expressed as the number of CFU (colony-forming units) which grew on MA were in the range of 10^4–10^6/ml (Table 1). Overall, the viable bacterial counts increased in abalones fed on diets compared to the larval abalone without any food. Higher numbers of bacteria were found in the gut homogenates of abalones fed on *Gracilaria* sp.

Thirty-three bacterial isolates with distinct phenotypes and RFLP profiles were obtained from abalone *H. diversicolor* from the four development stages (Table 2). 16S rDNA sequence analysis revealed that 33 strains could be allocated into four groups of Bacteria domain, 24 strains of γ-*Proteobacteria*, 5 strains of the family Bacillaceae belong to Firmicutes group, 3 strains of CFB group (*Cytophaga*/*Flexibacteria*/*Bacteroides*) and 1 strain of Sphingobacteria group. The Firmicutes group was isolated from the larvae and early juveniles, the CFB group was detected in the latter 3 stages, and the γ-Proteobacteria occurred in each development stage. Nine putatively novel bacterial strains were identified based on sequence similarity value below 98% to cultivated strains in the NCBI database.

At the larval stage, *Pseudoalteromonas* and *Bacillus* genera made up the main cultivated bacterial group. *Pseudoalteromonas* sp. was not isolated from abalone at other stages. *Bacillus* sp. were only isolated from the larvae and early juveniles. While in the early juvenile stage abalone, the *Bacillus* genus was not the dominant group. Another frequently encountered group, was found to consist of 18 strains (up to 54.55%, 18/33) affiliated with the family *Vibrionaceae*. This bacterial family was further divided into two genera, 16 *Vibrio* sp. and 2 *Photobacterium* sp. This most-abundant cluster was detected in the latter 3 stages, but absent in the larval stage. Members of the *Vibrio* genus showed 98–99% homology with *V. alginolyticus*, *V. harveyi*, *V. shilonii* or *V. tapetis*. The proportion of cultivated *Vibrio* genus in the gut's bacterial composition changed slightly among the different development stages. In the early-juvenile stage, a total of 64% of the bacterial isolates were identified as *Vibrio* and in the late-juveniles fed on artificial diets, 57% bacterial strains were also identified as *Vibrio* sp. In the gut homogenates from adult abalone, *Vibrio* species accounted for 40% of the cultivable microflora.

On the other side, the bacterial diversity increased with changing of the rearing diets. And some bacterial genera seem to be specific to one stage, such as *Pseudoalteromonas* in larvae, *Mucus* and *Flavobacteriales* in early-juveniles, *Agarivorans* and *Aquimarina* in late-juveniles, *Photobacterium*, *Tenacibaculum* and *Flammeovirga* in grow-out abalone (Fig. 1).

3.2. Analysis of bacterial flora with digestive enzyme activity

The cultivated gut bacteria showed different digestive enzyme activities at four stages with varied dietary conditions (Fig. 2 and Table A2). From the early juvenile (HE) abalone, the isolated bacterial flora and digestive enzymes showed the greatest diversities among four stages. All six digestive enzymes were detected in this stage where abalone was fed with diatom. It was noticeable that the alginate-degrading bacterial flora was second only to the bacterial lipase dominated in the HE stage. After 150 days of feeding diets supplemented with artificial food, the dramatic decrease with bacterial digestive enzymes occurred in the late-juvenile (HJ) stage. The alginate- and agar-degrading bacteria that dominated the HE stage, was not detected in the HJ stage. And the late-juveniles contained a very low percentage of bacterial community with digestive enzymes. After feeding on marine red seaweed for one year, both the diversity of digestive enzymes and the amount of bacteria with enzyme activities increased obviously. At the larval stage, the types of bacterial digestive enzyme were less diverse than those in other stages, which may due to the starvation and reliance on endogenous reserves.

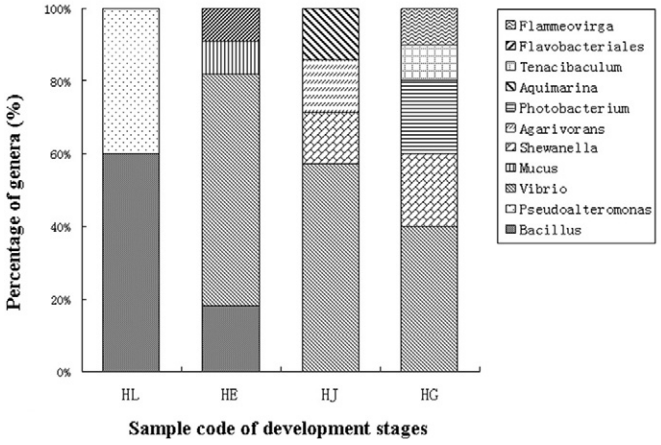

Fig. 1. The gut bacterial flora of abalone *H. diversicolor* at four development stages. HL, larva; HE, early juvenile; HJ, late juvenile; HG, grow-out.

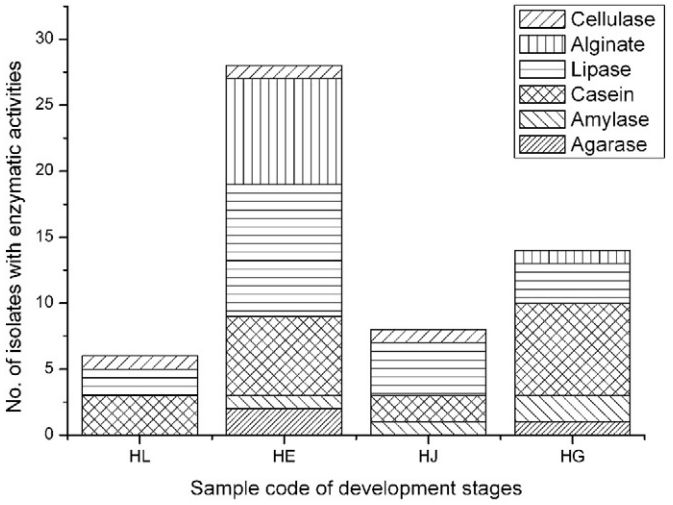

Fig. 2. The gut bacterial flora with digestive enzymes at four development stages of abalone *H. diversicolor*. HL, larvae; HE, early-juvenile; HJ, late-juvenile; HG, grow-out.

Compared with other bacterial enzymes, the bacteria with lipolysis and proteolysis activities might be the most predominant among four stages. At HE and HG stages, the amount of lipolytic bacteria was more than the casein-degrading bacteria. However, in the grow-out stage (HG), the result showed that the casein-degrading bacterial flora was more diverse than that in other stages. In this study, nearly 70% (10/15) *Vibrio* sp. could produce at least two digestive enzymes, particularly in the HE stage.

3.3. Comparison of the bacterial composition between abalones and diets

Eight samples were selected in DGGE analysis, which represented four development stages with distinctive diets (HL, HE, HJ and HG samples), their corresponding food and seawater samples. In the fingerprint profile, replicate treatments typically have the same fingerprint, thus confirming the reproducibility of the experimental treatments. The bacterial diversity in larval body and seawater showed unexpected abundance and similarity. Although rinsing repeatedly with sterile seawater, partial bacterial DNA fragments adhering to the external body were difficult to remove. The numbers of predominant fragments in the DGGE profiles were calculated for all samples (Table 3). The number of bands varied from 15 to 33 in abalone samples acquired from different development stages. Besides larvae, the total number of bands from early juveniles tended to be higher than those from late juveniles and grow-out. And the differences in number of DGGE bands in gut bacterial flora from juveniles or grow-out stages were not significant. The apparent richness of Bacterial flora was observed in artificial food fed samples (defined here as the number of bands on DGGE gels) seems to be the lowest in all samples.

Bacterial community structure, based on cluster analysis of the determinations of band position, showed clear differences among the four stages and consistency between the host and diet (Fig. 3).

Table 3
The number of DGGE bands in different samples.

Lane[a]	1	2	3	4	5	6	7	8	9
Num. of DGGE bands	33	33	35	35	25	25	20	22	5
Lane[a]	10	11	12	13	14	15	16	17	
Num. of DGGE bands	8	8	16	16	17	19	16	15	

[a] All lanes represent the bacterial flora from different samples, including 1–2, larva; lanes 3–4, sea water; 5–6, diatom; 7–8, early-juvenile; 9, marker; 10–11, artificial food; 12–13, late-juvenile; 14–15, red seaweed; 16–17, grow-out.

Dendrogram resulting from UPGMA method clustering based on genetic distance revealed that the 8 bacterial populations were divided into 3 branches. The bacterial flora associated with the larvae, seawater, early juveniles and diatom cluster into one branch, of which the early juveniles and its rearing food–diatom cluster on the first grade showing high similarity (up to 70%) between them. On the other cluster, the bacterial population derived from grow-out and the red seaweed *Gracilaria* sp. has higher homology, and formed the first grade. The bacterial communities associated the gut of grow-out abalone, *Gracilaria* sp. and the late juveniles cluster on the second grade showing high similarity (above 60%) among them. The bacterial population from artificial food was an independent distant from other branches.

4. Discussion

The intestinal microflora population is a complex ecosystem composed of a large variety of bacteria. Until recently there has been few detailed data about the extensive bacterial collections that exist in the mollusc's intestine. In this study, the cultured-dependent and -independent protocols were combined to identify the gut bacteria of the farmed abalone *H. diversicolor* through the development stage from larva to grow-out, investigate bacterial digestive enzymes with the change of diets, and analyze the correlation between the gut-associated flora and abalone.

During the non-feeding larvae, *Bacillus* sp. and *Pseudoalteromonas* sp. were presented as the greatest cultivated proportion compared to the latter three stages. This finding was in accordance with Atlantic halibut larva (Verner-Jeffreysa et al., 2003). Bacteria isolated from non-feeding yolk-sac larvae were predominantly non-fermentative Gram-negative rods, in particular *Pseudoalteromonas* species; by contrast, the presumptive gut microflora of first-feeding organisms was generally dominated by members of the *Vibrio* genus. Recent studies reported that marine *Pseudoalteromonas* species possessed broad range of bioactivity associated with the secretion of extracellular compounds (Sivasubramanian et al., 2011). Another genus *Bacillus* could be one of the important bacterial sources of polypeptide antibiotics (Ravi et al., 2007; Rengipipat et al., 2000). It has been isolated from the early juvenile marine organisms and tested as probiotics in marine aquaculture, such as shrimp (Watchariya et al., 2005) and fish (Lalloo et al., 2007). Considering that genus *Bacillus* and *Pseudoalteromonas* sp. could successfully colonize the gut and benefit host, it was presumed that establishment of intestinal bacteria was likely to develop "adult" microbiota excluding potential invaders and maintaining health before immune system maturation (Rawls et al., 2004).

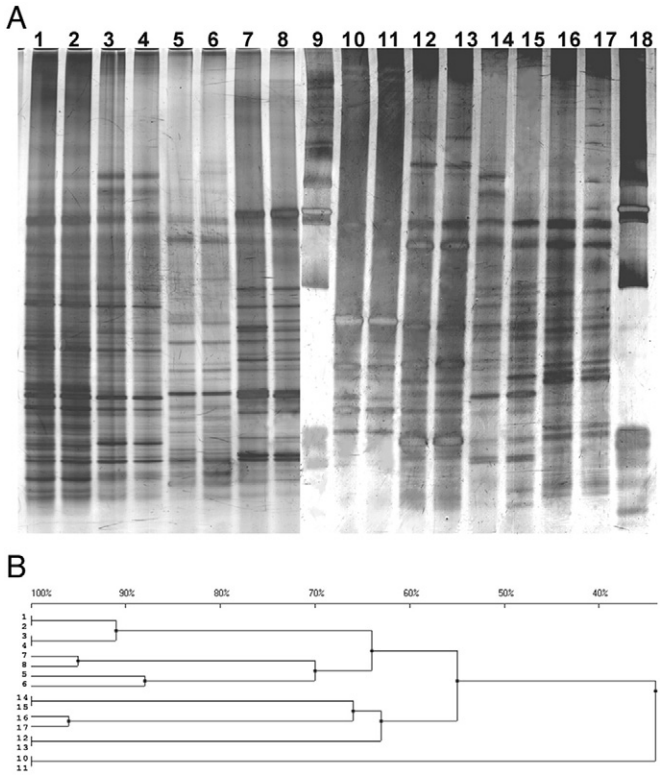

Fig. 3. Cluster analysis of DGGE profile based on quantification of the band position and intensity. A, DGGE profile of gut bacterial diversity in four development stages, seawater, and different diets. B, Clustering dendrogram of all samples. The scale bars indicate the percentage of Pearson correlation in all samples. All lanes represent the bacterial flora from different samples, including 1–2, larva; 3–4, sea water; 5–6, diatom; 7–8, early-juvenile; 9, marker; 10–11, artificial food; 12–13, late-juvenile; 14–15, red seaweed; 16–17, grow-out.

Following the onset of feeding, the abalone were colonized by *Vibrio* species originating from their diatoms with metabolically activities, particularly protease, lipase, alginate-degrading enzyme. It indicated that the *Vibrio* genus could be foreign bacteria which entered the gastrointestinal of abalone through diets from juvenile to adult and produced main bacterial digestive enzymes (Tanaka, et al., 2003; Tanaka et al., 2004). The genus *Vibrio* has been considered the major pathogenic strains associated with the marine invertebrates (Cai et al., 2006). In this study, most *Vibrio* species isolated from the latter three stages were involved with the mass mortality of abalone, clam, fish or other marine life (Hidalgo et al., 2008; Jensen et al., 2003; Liu et al., 2001). However our previous study with molecular methods showed that *Vibrio* sp. was the major group in healthy abalone *H. diversicolor* (Huang et al., 2010). Macey also has found that *Vibrio midae* from the intestine of abalone *H. midae* could degrade varied polysaccharides and affect favorably various gut microbiota by stimulating the digestive activities (Macey and Coyne, 2006). These results corroborate that most *Vibrio* sp. maybe beneficial to the host in nature conditions, and abalone mortalities could not be associated with primary or specific *Vibrio* pathogens, but rather with opportunistic or indigenous bacteria that attack the host abalone under stress conditions or inadequate microbial conditions (Sawabe et al., 1998).

DGGE profile indicated that the change and succession in the microbial composition existed in four growth stages. Some microorganisms might be transient and subject to environmental factors, such as different dietary style. The bacterial flora from the abalone intestine and food clustering within the similar phylogenetic clade further confirmed this point. On the contrary, the bacterial composition from the abalone remained a succession associated with live food, and this succession could be interrupted by an artificial diet. Although artificial food provides essential nutrients to support growth and development of the abalone, they also could alter the intestinal microbial ecosystem of the host organism (Burr et al., 2005). Assays of the digestive enzymes from cultivated bacteria further showed that the farmed *H. diversicolor* intestine of late juveniles carried a unique collection of trillions of microbes. Many studies have reported that the balance with gut microflora influences the innate immune system, particularly in invertebrates lacking specific immune system, which is of vital importance for the disease resistance (Gómez and Balcázar, 2008; Husseneder et al., 2009; Jaenike et al., 2010). Thus, it implied that constantly changing diet, especially the introduction of artificial food could disturb the succession of gut microflora. The interruption of microbial stability may increase the occurrence of feed associated disease outbreaks in aquaculture animals (McIntosh et al., 2008; Round and Mazmanian, 2009). From another angle, the late-juvenile stage may be an appropriate period in which probiotic supplements with artificial diet are fed to *H. diversicolor* to affect gut microbial flora.

The results of enzyme assay indicated that intestinal microflora potentially played an important role in harvesting energy from food throughout all development stages. Comparison of the diversity of bacterial digestive enzymes from abalone intestine showed that different species of abalone involve distinctive exogenous enzymes. In abalone *H. midae*, bacterial

agarase and carrageenase enzyme activities were most pronounced (Erasmus et al., 1997). Dominant species were alginolytic, non-motile fermenters in *H. discus hannai* (Tanaka et al., 2003). In the farmed abalone *H. diversicolor*, the bacterial casein, lipase and alginate lyase activities were higher, especially in the early-juvenile abalone. It implied that bacteria in the feeding habit and surrounding environment might have profound influence on the composition of the gut-associated flora in abalone (Hehemann et al., 2010). In addition, host may acquire digestive enzymes from gut bacteria, especially to digest those substrates which few animals can digest, such as cellulose, alginate (Kar and Ghosh, 2008). So, the bacterial enzymes also further reflect the close link between the gut bacterial flora and diets (Hansen and Olafsen, 1999).

It is well recognized that most bacteria have not been cultured yet in the laboratory because what their growth require are still unknown. But the cultivation is indispensable for functional analysis of bacteria, such as enzymatic activity (Kar and Ghosh, 2008). Therefore, it is important to apply both the culture-based and culture-independent techniques into the study. Similar results with consistent tendency were acquired from these two methods, including the succession of bacterial flora among different development stages, the change of gut flora accompanied with the shift of diets, particularly the artificial food may break the natural succession or balance of the bacterial community. In conclusion, all information might contribute to further understanding the interaction between microbe and host abalone, and controlling the bacterial flora to improve the resistance of abalone against pathogen. However, further research involving uncultured bacterial strains should be conducted to describe the gut-associated flora of marine invertebrates under actual farm conditions in future.

Policy and ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of Fujian, China (No. 2009J01191); Hi-Tech Research and Development (863) Program of China (No. 2012AA100812); the Earmarked Fund for Modern Agro-industry Technology Research System (No. CARS-48). Professor John Hodgkiss is thanked for his assistance with English.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.aquaculture.2012.01.016.

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