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Gut microbiota of fast and slow growing grouper *Epinephelus coioides*

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Although the gut microbiota of fish has been studied extensively using traditional or molecular techniques, little information is available on the correlation between gut microbiota and growth of host fish. In the present study, the gut microbiota of two groups of juvenile grouper *Epinephelus coioides*, slow growing (SG) and fast growing (FG) grouper at 75 days post-hatch (DPH), were investigated by using standard isolation and characterization procedures. The results showed that 4 *Vibrio* species were isolated and comprised 12.3% of the total gut bacteria in SG grouper, whereas only two *Vibrio* species were isolated and comprised 3.6% of the total bacteria in FG grouper. At the same time, *Bacillus pumilus*, *Bacillus clausii* and *Psychroacter* sp. were only isolated and dominated in the gut of FG fish. The 3 species showed antagonistic effect on pathogenic *Vibrio*, this may cause the lower number and less species of *Vibrio* in the gut of FG grouper and suggesting fast growing fish might harbor a favorable microbiota. The number of total cultivable aerobic and facultative anaerobic bacteria was similar in the gut of two groups, 5.4×10^6 CFU/g and 9.0×10^6 CFU/g in SG and FG group, respectively. Besides those most common bacteria such as *Vibrio* spp., *Pseudomonas* spp. and *Acinetobacter* spp., several Gram-positive and Gram-negative bacteria not normally in the gut of marine fish were also isolated from both groups of fish. The bacteria in the gut of grouper could be classified into three groups belonging to -proteobacteria, -proteobacteria and Bacilli class.

Key words: Gut microbiota, fast growing grouper, slow growing grouper, *Epinephelus coioides*

INTRODUCTION

The gastrointestinal (GI) tract of fish is a complex ecosystem possessing a specific microbiota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria (Cahill, 1990; Gómez and Balcázar, 2008) . The composition has been suggested to change with host age, nutritional status, and environmental conditions (Conway et al., 1986; Eddy and Jones, 2002; Verner-Jeffreys et al., 2003), but generally a primary transient microbiota is established at the larval stage, developing into a relatively stable flora at the juvenile stage or after metamorphosis (Olafsen, 2001; Eddy and Jones, 2002). Compared to human being and warm-blooded animals, the roles of gut microbiota in fish are poorly understood. In general, the gut microbiota has been suggested to

hinder the colonization of pathogenic bacteria (Kennedy et al., 1998; Verschuere et al., 2000; Spanggaard et al., 2001), stimulate immune response (Olafsen, 2001; Gómez and Balcázar, 2008), or produce some beneficial bioactive substances such as essential fatty acids (Ringø et al., 1992), vitamins (Sugita et al., 1991), digestive enzymes (Bairagi et al., 2002; Skrodenyt - Arba iauskien , 2007) and antibacterial substances (Sugita et al., 1998; 2002). Therefore, it is generally accepted that there is a possible symbiotic relationship between fish and gut microbiota (Verschuere et al., 2000).

During rearing, the growth rate of fish varies greatly and can be affected by a variety of factors such as tem-perature, water quality, territorial defense, unnatural habi-tats, stocking densities and available nutrition (Sumpter, 1992; Baltz et al., 1998). However, under identical rearing conditions, the growth rate may be affected by "internal factors" such as central nervous system, endocrinological and neuroendocrinological systems (Boeuf et al., 1999).

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In addition, the gut microbiota could be a "new" possible "internal factor" as it is gradually recognized that it plays very important role in the health and growth of the host (Vine et al., 2006; Comstock, 2007; Mazmanian et al., 2008). Presumably, therefore, fast growing fish might harbor a more favorable gut microbiota. However, to the authors knowledge only one study has compared the gut microbiota of dominant (fast growing) and subordinate (slow growing) individuals of Arctic charr, *Salvelinus alpinus* (L.), and the results showed some difference in the gut microbiota between dominant and subordinate individuals (Ringø et al., 1997).

Grouper is an important marine fish species and has been used in commercial rearing in China and Southeast Asian countries for its excellent biological characteristics, such as fast growth, disease resistance, popular taste and high economic value (Yeh et al., 2003). In the present study, we investigated the gut microbiota of fast growing (FG) and slow growing (SG) grouper *Epinephelus coioide* by using standard isolation and characterization procedures in combination with 16S rRNA gene sequence analysis as Ringø et al. (2006b) and Spanggaard et al. (2000).

MATERIALS AND METHODS

Fish and rearing condition

Fertilized eggs of grouper were hatched in a private hatchery in Zhangpu, Fujian province, China. The larvae were cultured in 4 × 6 m indoor concrete ponds. The feeding scheme was as follows: fertilized eggs of the Pacific oyster *Crassostrea gigas* were used as diet for first feeding from 3 to 7 DPH. from 7 to 21 DPH, larvae were fed enriched Rotifer *Brachionus plicatilis*; from 15 - 21 DPH, *Artemia* nauplii (*Artemia salina*) were introduced; from 21 - 40 DPH, larvae were fed Copepod and *Artemia* nauplii; From 40 - 75 DPH, juvenile grouper were fed *Artemia* adult and frozen fish meat. At 1600 h, the debris on the bottom of the ponds was sucked out with a plastic hose, and then the ponds were refilled with clean seawater. Water temperature and salinity during the experiment were 26 - 31°C and 30 - 35 g/l, respectively. Larvae were first graded and divided into two groups at 36 DPH using the No. 1 grader (Hseu, 2004), those passed through the grader (Total length < 2.73 cm) were put into one pond and those remained (Total length 2.73 cm) were put into another pond. After this, the larvae were routinely graded and put into different ponds at 12 days intervals using a series of graders (Hseu, 2004). After the last grading at 72 DPH, four groups of fish with different size were put into four different ponds. To study the gut microbiota of fast and slow growing fish, two groups of fish at 75 DPH with biggest difference in growth rate were selected for gut microbiological study, one is slow growing group (SG, 5.1 ± 0.4 cm long and 5.2 ± 0.5 g weight), the other is fast growing group (FG, 8.1 ± 0.6 cm long and 10.0 ± 1.1 g weight).

Isolation of gut bacteria

Five fish of each group and approximately 100 ml of inlet water was sampled before morning feeding for microbiological analysis. Fish was killed by a sharp blow on the head and dissected under aseptic conditions, fat deposits surrounding the GI tract were removed, and

the GI tract with content was weighted individually (0.4 ± 0.1 g and 1.0 ± 0.2 g for SG and FG group, respectively). To avoid individual variations of the gut microbiota (Spanggaard et al., 2000), the GI tracts with content of 5 fish were pooled and homogenized in 10 ml of a sterile nine-salt solution (NSS) (Olsson et al., 1992) . Gut homogenates and water sample were diluted in NSS and appropriate dilutions were spread on the surface of marine agar (MA) plates (Hopebio, Qingdao, China), Thiosulphate-Citrate- Bile Salt- Sucrose (TCBS) plates (Land bridge, Beijing, China) and De Man Rogosa and Sharpe (MRS) plates (Land bridge, Beijing, China) in duplicate, respectively. MA was used to estimate the total cultivable aerobic and facultative anaerobic bacteria, TCBS was used to estimate genus *Vibrio*, while MRS was used to estimate lactic acid bacteria (LAB) . The plates were incubated at 28°C and inspected regularly for up to 2 weeks and the total bacterial colonies were counted. The bacterial colonies were divided into different types according to the result of Gram staining and the colony characteristics of shape, size, elevation, structure, surface, edge, color and opacity, the number of colonies of each recognizable type was counted. Three to five representatives of each colony type were then streaked on corresponding plates repeatedly until pure cultures were obtained. A total of 109 isolates (41 from the gut of FG fish, 43 from the gut of SG fish and 25 from water) were tested physiological and biochemical properties, such as cell shape, pigmentation, Gram stain, spores and encapsulation, ability to growth on TCBS, sugar fermentation and so on (Ringø et al., 1997). A total of 20 (13 from the gut of FG fish, 4 from the gut of SG fish and 3 from inlet water) representative isolates were characterized further by 16S rRNA gene analysis according to Sun et al. (2008).

16S rRNA gene sequencing and Phylogenetic analysis

Pure bacterial culture was cultivated in nutrient broth (10 g peptone; 3 g beef extract and 5 g sodium chloride to 1 L deionized water) for 2 d at 28°C, and then the culture was centrifuged for 10 min at 5031 × g. A total of 10 - 20 mg of bacterial precipitate was placed into a 1.5 ml microcentrifuge tube and resuspended in 200 l of TE buffer. Bacterial DNA was extracted using Bacterial Genomic DNA Purification Kit (Tiangen, Beijing, China). The following PCR and sequencing was conducted as described in Sun et al. (2008). Homology searches of the GenBank DNA database was performed with BLAST Search. The sequences of 17 strains from the gut of grouper were aligned by CLUSTALX version 1.83 (Thompson et al., 1997) with those of corresponding sequences from other bacteria in the GenBank databases by BLAST search. A phylogenetic tree was constructed by the neighbor -joining method (Felsenstein, 2005) on paired alignment of nucleic acid sequences of the bacterial 16S rRNA gene. The sequences from this study have been deposited in the GenBank databases under accession numbers EU520326-EU520345.

Inhibition assay

Bacillus pumilus, *Bacillus clausii* and *Psychrobacter* sp. were only isolated and dominated in the gut of FG grouper, while lower number and less species of pathogenic *Vibrio* in the gut of FG grouper (Table 1). We speculated that *B. pumilus*, *B. clausii* and *Psychrobacter* sp. may have antagonistic effect on *Vibrio*. Therefore, *B. pumilus*, *B. clausii* and *Psychrobacter* sp. were tested for antagonistic activity in a well diffusion agar assay (WDAA) against the 4 *Vibrio* species in the gut of grouper. The *Vibrio* was grown in 4 ml nutrient broth for 2 d at 28°C, subcultured in nutrient broth for 1 d at 28°C, and 300 ul of each pathogenic bacterium cultures was mixed into 10 ml of melted (43.5 - 44°C) nutrient broth agar. After solidifying and drying for 15 - 20 min, wells were punched (diameter=3 mm) and 10 l of 2 d old *B. pumilus*, *B. clausii*

Table 1. Bacterial number on MA, TCBS and MRS plates from inlet water, the gut samples of slow growing (SG) and fast growing (FG) grouper.

Media	SG (CFU/g)	FG (CFU/g)	Inlet water (CFU/ml)
MA	5.4×10^6	9.0×10^6	5.4×10^5
TCBS	6.0×10^5	3.4×10^5	2.2×10^3
MRS	1.5×10^5	6.0×10^4	-

MA, marine agar; TCBS, Thiosulphate-Citrate-Bile Salt-Sucrose; MRS, De Man Rogosa and Sharpe. CFU, colony forming unit.

and *Psychrobacter* sp. culture (approx. 10^8 - 10^9 CFU/ml) grown in nutrient broth was introduced into the wells. The diameter of inhibitory zone formed around the well after 48 h of incubation at 28°C was recorded. Each assay was performed in triplicate.

Statistical analysis

Fish length, fish weight, gut weight and inhibitory assay data were analyzed by one-way of analysis of variance (ANOVA) followed by Duncan's multiple comparison procedure using the statistical packages for the Social Sciences11.5 for windows (SPSS, Inc., Chicago, IL). Bacterial number was a mean of bacterial counts in duplicate plates.

RESULTS

Bacterial enumeration

The total cultivable aerobic and facultative anaerobic bacteria on MA plates in SG group was (5.4×10^6 CFU/g) lower than that in FG group (9.0×10^6 CFU/g) (Table1), but the number of *Vibrio* (bacteria on TCBS plates) in SG group (6.0×10^5 CFU/g) was higher than that in FG group (3.4×10^5 CFU/g) (Table 1). Also, the number of lactic acid bacteria (bacteria on MRS plates) in SG group (1.5×10^5 CFU/g) was higher than that in FG group (6.0×10^4 CFU/g). Inlet water harbors a much lower number of bacteria (5.4×10^5 CFU/g) than the gut of grouper, and lactic acid bacteria were not found in inlet water (Table 1).

Species composition of bacterial isolates

As shown in Table 2, a wide range of bacterial species were isolated from the gut of two groups of fish, and the relative abundance of bacterial genera was presented in Figure 1. Eleven species existed in the gut of both groups of fish that is *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Delftia acidovorans*, *Pseudomonas putida*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Erwinia carotovora*, *Staphylococcus aureus*, *Lactococcus lactis*, *Lactobacillus casei* and *Enterococcus faecium*. However, *Vibrio metschnikovi* and *Vibrio alginolyticus* were isolated only from SG fish, whereas *Bacillus pumi-*

lus, *Bacillus clausii* and *Psychroacter* sp. only from FG fish (Table 2). Interestingly, 4 species of *Vibrio*, *V. parahaemolyticus*, *V. harveyi*, *V. metschnikovi* and *V. alginolyticus*, were isolated from the gut of SG group, with number ranged from 7.5×10^4 to 3.7×10^5 CFU/g, but only two species of *Vibrio* , *V. parahaemolyticus* and *V. harveyi*, were isolated from FG group.

The predominant bacteria of two groups showed huge variation, *Delftia, Acinetobacter*, *Vibrio* and *Pseudomonas* were the predominant bacteria in SG group, comprising 48.1, 18.1, 12.3 and 12.0% of the total gut bacteria respectively, while *Bacillus*, *Delftia*, *Acinetobacter* and *Psychrobacter* were the predominant bacteria in SG group, comprising 37.8, 26.7, 13.3 and 11.1% of the total gut bacteria (Figure 1). Among those predominant bacteria, *Delftia*, *Acinetobacter* and *Pseudomonas* in two groups were almost equal in number (Table 2). However, *Vibrio* as one of predominant bacteria (12.3%) in SG group was only comprised 3.6% of the total bacteria in FG group (Figure 1).

Phylogenetic analysis showed that 16S rRNA gene sequences of 17 representative gut isolates could be classified into three groups belonging to -proteobacteria, -proteobacteria and Bacilli class (Figure 2). For -proteobacteria, three strains (ST4, ST6 and ST7) were clustered within the *Vibrio* group, two strains (ST1 and ST5) within the *Acinetobacter* group; strain SE6 showed high homology to *Psychrobacter* sp. and was only detected in FG group; the remaining two strains (SE3 and MM6) were most closely related to *P. putida* and *E. carotovora*, respectively. For -proteobacteria, strain SE4 was the most abundant bacterium in SG group, with high homology to *D. acidovorans*; strain ST2 was phylogenetically related to *B. cepacia.* For Bacilli, strain SE5 and DE5 were phylogenetically related to *B. pumilus* and *B. clausii*, which were only detected in FG group; three strains (SE1, MM5 and DM1) were clustered within the *Staphylococcus* group; strain MM1 and MM4 were most closely related to *L. lactis* and *E. faecium*.

In water, only 9 species were isolated, including *B. pumilus*, *B. clausii*, *V. parahaemolyticus*, *V. harveyi*, *V. metschnikovi*, *A. baumannii*, *Psychrobacter* sp., *B. cepacia* and *Nocardioides* sp. Among those bacteria, *Bacillus* and *Psychrobacter* were the most predominant bacteria.

Inhibition of potential beneficial bacteria to suspected pathogenic *Vibrio*

As showed in Table 3, *B. pumilus*, *B. clausii* and *Psychrobacter* sp. showed antagonistic effect on three of the four *Vibrio* species. *B. pumilus* could inhibit *V. harveyi*, *V. metschnikovi* and *V. alginolyticus* , while *B. clausii* could inhibit *V. parahaemolyticus*, *V. metschnikovi* and *V. alginolyticus*; *Psychrobacter* sp. showed antagonistic effect on *V. harveyi*, *V. metschnikovi* and *V. alginolyticus*.

Table 2. Bacterial composition in the gut of slow growing (SG) grouper, fast growing (FG) grouper and inlet water.

Strain	Accession number	SG (CFU/g)	FG (CFU/g)	Inlet water (CFU/ml)
Bacillus pumilus	EU520340	n.d.	1.8×10^6	1.4×10^5
Bacillus clausii	EU520331	n.d.	1.6×10^6	1.6×10^5
Vibrio parahaemolyticus	EU520337	1.2×10^5	2.6×10^5	1.2×10^3
Vibrio harveyi	EU520336	3.7×10^5	6.0×10^4	1.6×10^3
Vibrio metschnikovi*		7.5×10^4	n.d.	2.9×10^3
Vibrio alginolyticus*		1.0×10^5	n.d.	n.d.
Delftia acidovorans	EU520344	2.6×10^6	2.4×10^6	n.d.
Pseudomonas putida	EU520339	6.5×10^5	5.0×10^5	n.d.
Acinetobacter baumannii	EU520338	9.8×10^5	1.4×10^6	1.5×10^3
Psychrobacter sp.	EU520334	n.d.	1.2×10^6	2.2×10^5
Burkholderia cepacia	EU520342	1.5×10^4	3.0×10^3	60
Erwinia carotovora	EU520329	1.9×10^4	7.5×10^3	n.d.
Staphylococcus aureus	EU520330	2.4×10^5	1.4×10^4	n.d.
Lactococcus lactis	EU520326	2.5×10^4	1.0×10^4	n.d.
Lactobacillus casei*		8.0×10^4	3.9×10^4	n.d.
Enterococcus faecium	EU520327	3.8×10^4	1.4×10^4	n.d.
Nocardioides sp.	EU520345	n.d.	n.d.	1.0×10^3

*Not identified by 16S rRNA gene sequencing; n.d. not detected.
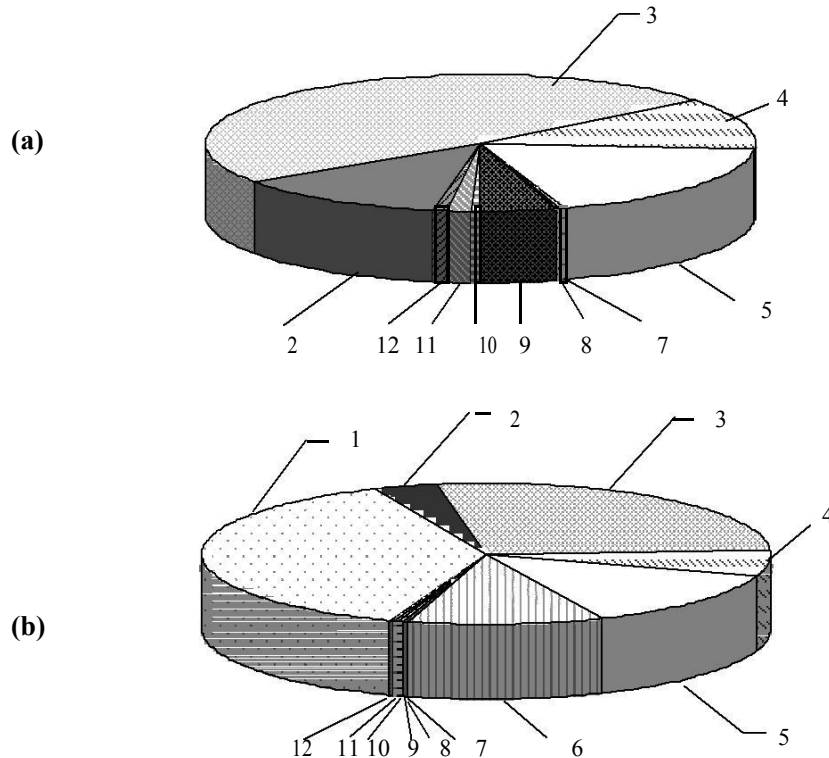

Figure 1. Relative abundance of bacterial genera in the gut of small grouper (a) and large grouper (b). (a) 2, *Vibrio* 12.3%; 3, *Delftia* 48.1%; 4, *Pseudomonas* 12.0%; 5, *Acinetobacter* 18.1%; 7, *Burkholderia* 0.3%; 8, *Erwinia* 0.4%; 9, *Staphylococcus* 4.4%; 10, *Lactococcus* 0.5%; 11, *Lactobacillus* 1.5%; 12, *Enterococcus* 0.7%. (b) 1, *Bacillus* 37.8%; 2, *Vibrio* 3.6%; 3, *Delftia* 26.7%; 4, *Pseudomonas* 5.6%; 5, *Acinetobacter* 13.3%; 6, *Psychrobacter* 11.1%; 7, *Burkholderia* 0.03%; 8, *Erwinia* 0.08%; 9, *Staphylococcus* 0.2%; 10, *Lactococcus* 0.1%; 11, *Lactobacillus* 0.4%; 12, *Enterococcus* 0.2%.
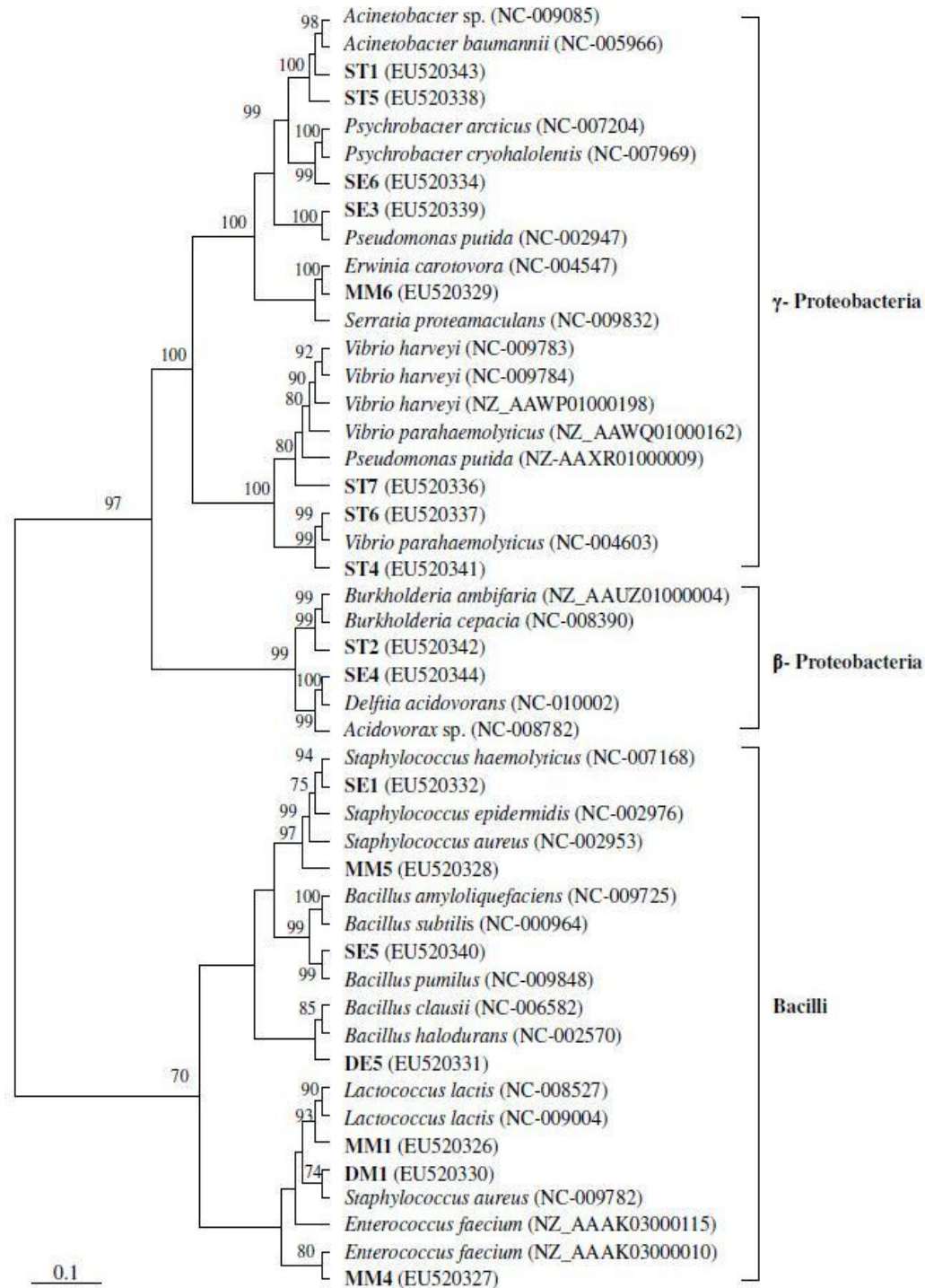
Figure 2. Phylogenetic tree was constructed by the neighbor-joining method based on the partial sequence of the 16S rRNA gene for the bacteria isolated from the gut of grouper and corresponding region in those for the authentic bacterial genes. Numbers at branches denote the bootstrap percentages of 1000 replicates. Only bootstrap values >70% are indicated. The scale at the bottom indicates the evolutionary distance of nucleotide substitutions per site. EU520326-EU520345 is the accession number deposited in the GenBank databases in the present study and the accession number for reference sequences are shown in parenthesis.

Among those *Vibrio*, *V. metschnikovi* and *V. alginolyticus* were inhibited by all the three potential beneficial

Table 3. Antagonistic activity of potential beneficial bacteria against pathogenic *Vibrio*.

Pathogenic *Vibrio*	Diameter of inhibitory zone (mm)		
	Bacillus pumilus	*Bacillus clausii*	*Psychrobacter* sp.
Vibrio parahaemolyticus	0	9.9 ± 0.5	0
Vibrio harveyi	10.3 ± 1.0	0	9.6 ± 0.8
Vibrio metschnikovi	10.4 ± 1.1	9.0 ± 0.6	8.2 ± 0.3
Vibrio alginolyticus	11.1 ± 0.8	9.3 ± 0.8	9.0 ± 0.4

bacteria.

DISCUSSION

In contrast to terrestrial animals, fish have closer contact with the environmental microbiota due to their aqueous habitat. Surrounding bacteria are continually ingested with food or water. For this reason, transient micro-organisms probably have a more constant and important interaction with fish gastrointestinal ecosystems compared to terrestrial animals. In general, genus *Acinetobacter*, *Vibrio* and *Pseudomonas* were the most common bacteria in the gut of marine fish (Muroga et al., 1987; Cahill, 1990; Munro et al., 1994; Gatesoupe et al., 1997), and *Vibrio* was considered as one of the predominant bacteria (Gatesoupe et al., 1997; Olafsen, 2001; Eddy and Jones, 2002; Sugita and Ito, 2006). In the present study, the microbiota in the gut of two groups of grouper *Epinephelus coioides* including both allochthonous and autochthonous bacteira were studied. The results showed that *Acinetobacter*, *Vibrio* and *Pseudomonas* were also isolated from the gut of both group of fish (Figure 1). However, *Vibrio* dominated only in the gut of SG fish (12.3%), whereas comprising 3.6% of the total bacteria in FG fish. In addition, four *Vibrio* species, *V. parahaemolyticus*, *V. harveyi*, *V. metschnikovi* and *V. alginolyticus* appeared in SG group, but only two *Vibrio* species (*V. parahaemolyticus* and *V. harveyi*) in FG group. The exact mechanism behind this variation was unclear, but antagonism among gut bacteria could be a possible reason. In the present study, the dominant *B. clausii* and *Psychrobacter* sp. were only isolated and dominated in the gut of FG group and showed antagonistic effect on *Vibrio* species (Table 3), this may cause the low number of *Vibrio* in the gut of FG fish. Especially, *B. pumilus*, *B. clausii* and *Psychrobacter* sp. showed a good antago-nistic effect on *V. metschnikovi* and *V. alginolyticus*, this may cause the disappearance of the two *Vibrio* species in the gut of FG fish. This was in agreement with an in vivo study by Kennedy et al. (1998) who observed that *Bacillus* sp. isolated from marine fish with antagonistic effect on pathogens could exclude pathogenic *Vibrio* from common snook (*Centropomus undecimalis*) . As the four *Vibrio* species were considered as common opportunistic pathogens of marine fish and gut as their main infection

site (Diggles et al., 2000; Ringø et al., 2007), this relatively lower number and less bacteria of two groups showed huge variation, *B. pumilus*, species of pathogenic *Vibrio* in the gut of FG group would likely to reduce the potential risk to the host (Olafsen, 2001; Vine et al., 2006).

B. pumilus, *B. clausii*, *D. acidovorans*, *A. baumannii* and *Psychrobacter* sp. were the predominant bacteria in the gut of FG grouper, presumably, those bacteria are likely to be related with the health and growth of host for their high number (Cahill, 1990). However, *D. acido-vorans* and *A. baumannii* have been identified in human clinical isolates (Horowitz et al., 1990; Bofill et al., 1996; Bergogne-Berenzin and Towner, 1996), and *A. baumannii* could even cause severe infections in mandarin fish (Gu et al., 1997). Therefore, the role of *D. acidovorans* and *A. baumannii* in the gut of grouper need further study. the three species dominated in inlet were only isolated and dominated in FG group although Interestingly, *B. pumilus*, *B. clausii* and *Psychrobacter* sp water, their roles in the gut of fish are therefore worth our attention.

Bacillus has been successfully isolated from the gut of. several marine fish and applied as probiotics (Kennedy et al., 1998; Velmurugan and Rajagopal, 2009), but few studies showed it was the predominant bacteria (Martin-Antonio et al., 2007; Hovda et al., 2007). In the present study, *B. pumilus* and *B. clausii* were the most dominant bacteria in the gut of FG group and demonstrated antagonistic effect on pathogenic *Vibrio* (Table 3). Previous studies have showed that many *Bacillus* strains isolated from marine fish could inhibit potential pathogens (Sugita et al., 1998; Kennedy et al., 1998) and one strain (*Bacillus* no. 48) has been successfully used as pro-biotics to exclude pathogenic *Vibrio* from common snook (*Centropomus undecimalis*) (Kennedy et al., 1998). Therefore, it is reasonable that *B. pumilus* and *B. clausii* with antagonistic effect on pathogenic *Vibrio* are likely to play important role in the health of host fish although further study in needed.

Recently, *Psychrobacter* has been successfully isolated from the gut of marine fish, such as Arctic charr (*Salvelinus alpinus* L.) (Ringø et al., 2006a) and Atlantic cod (*Gadus morhua* L.) (Ringø et al., 2006b), but its role in the gut of fish is unclear. In the present study, *Psychro-bacter* sp. was only isolated and dominated in the gut of FG group, and the strain could inhibit 3 suspected patho-

genic *Vibrio* (Table 3). Therefore, we presumed that the species may play positive role in health of fish although further study is needed to be carried out.

To our knowledge, this is the first report of gut microbiota of juvenile grouper *Epinephelus coioides*. Therefore, the number and composition of gut microbiota are discussed below. Total bacterial number of SG and FG appeared on MA was similar, 5.4×10^6 and 9.0×10^6 CFU/g respectively (Table 1). These values fall within the range described for other juvenile marine fish, such as Japanese flounder *Paralichthys olivaceus* (3.6×10^5 - 6.0×10^7 CFU/g) (Sugita et al., 2002) and Senegales sole *Solea senegalensis* (2.3×10^5 - 6.7×10^6 CFU/g) (Martin-Antonio et al., 2007), but higher than that of juvenile Coho salmon *Oncorhynchus Kisutch* (6.0×10^3 CFU/g) (Romero and Navarrete, 2006). Several factors, such as bacterial host specificity (Cerdà-Cuéllar and Blanch, 2002), food type (Eddy and Jones, 2002) and water resource (Verner-Jeffreys et al., 2003) may explain these differences.

Both marine and freshwater fish have been shown to have a specific indigenous gut microbiota (Olafsen, 2001; Vine et al., 2006) and it may change with fish age, nutriational status, and environmental conditions (Olafsen, 2001). In line with previous reports (Muroga et al., 1987; Cahill, 1990; Munro et al., 1994; Gatesoupe et al., 1997), those most common genera such as *Vibrio*, *Pseudomonas* and *Acinetobacter* were also isolated from the gut of *Epinephelus coioides* . Interestingly, besides Gram-positive *L. lactis* and *E. faecium*, several "new" Gram-negative bacteria not normally in the gut of fish were also isolated and identified, such as *D. acidovorans*, *Psychrobacter* sp., *B. cepacia* and *E. carotovora*. Phylogenetic analysis showed that the gut microbiota of grouper could be classified into three groups belonging to -proteobacteria, -proteobacteria and Bacilli class (Figure 2), which was similar with the results of Coho salmon *Oncorhynchus Kisutch* (6.0×10^3 CFU/g) (Romero and Navarrete, 2006) and Senegales sole *Solea senegalensis* (2.3×10^5 - 6.7×10^6 CFU/g) (Martin-Antonio et al., 2007).

In summary, the number of gut bacteria in fast growing grouper and slow growing grouper *Epinephelus coioides* was similar, but the composition showed some difference, lower number and less species of pathogenic *Vibrio* was in the gut of fast growing grouper, while *Bacillus* and *Psychrobacter* with antagonistic effect on pathogenic *Vibrio* were only isolated and dominated in the gut of fast growing grouper. This may suggest that fast growing grouper might harbor a more favorable microbiota.

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REFERENCES

Bairagi A, Ghosh KS, Sen SK, Ray AK (2002). Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacul. Int. 10: 109-121.

Baltz DM, Fleeger JW, Rakocinski CF, McCall JN (1998). Food, density, and microhabitat: factors affecting growth and recruitment potential of juvenile saltmarsh fishes. Environ. Biol. Fish. 53: 89–103.

Bergogne-Berenzin E, Towner KJ (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin. Microbiol. Rev. 9: 148-165.

Boeuf G, Boujard D, Ruyet JPL (1999). Control of the somatic growth in turbot. J. Fish Biol. 55: 128-147.

Bofill L, Wessolossky M, Vicent E, Salas M, Besso J, Merentes A, Istúriz R, Guzmán M, Murillo J (1996). Septic shock due to *Comamonas acidovorans*: a most unusual association. Infect. Dis. Clin. Pra. 5: 73-74.

Cahill MM (1990). Bacterial flora of fishes: a review. Microb. Ecol. 19: 21-41.

Cerdà-Cuéllar M, Blanch AR (2002). Detection and identification of *Vibrio scophthalmi* in the intestinal microbiota of fish and evaluation of host specificity. J. Appl. Microbiol. 93: 261-268.

Comstock LE (2007). Microbiology: The inside story. Nature 448: 542-544.

Conway PL, Maki J, Mitchell R, Kjelleberg S (1986). Starvation of marine flounder, squid and laboratory mice and its effect on the intestinal microbiota. FEMS. Microbiol. Lett. 38: 187–195.

Diggles BK, Carson J, Hine PM, Hickman RW, Tait MJ (2000). Vi*brio* species associated with mortalities in hatchery-reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in New Zealand. Aquac. 183: 1-12.

Eddy SD, Jones SH (2002). Microbiology of summer flounder *Paralichthys dentatus* fingerling production at a marine fish hatchery. Aquac. 211: 9-28.

Felsenstein J (2005). PHYLIP (Phylogeny Inference Package) version 3.63 [computer program]. Department of Genome Science, University of Washington, Seattle, USA.

Gatesoupe FJ, Infante JLZ, Cahu C, Quazuguel P (1997). Early weaning of seabass larvae, *Dicentrarchus labrax*: the effect on microbiota, with particular attention to iron supply and exoenzymes. Aquac. 158: 117-127.

Gómez GD, Balcázar JL (2008). A review on the interactions between gut microbiota and innate immunity of fish. FEMS. Immunol. Med. Microbiol. 52: 145-154.

Gu T, Lu C, Chen H (1997). *Acinetobacter baumannii* a novel pathogen of acute epidemic in Mandarin fish (*Siniperca chuatsi*). Microbiology 24: 104-106.

Horowitz H, Gilroy S, Feinstein S, Gilardi G (1990). Endocarditis associated with *Comamonas acidovorans*. J. Clin. Microbiol. 28: 143-145.

Hovda MB, Lunestad BT, Fontanillas R, Rosnes JT (2007). Molecular Characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). Aquac. 272: 581-588.

Hseu JR (2004). The separating effect of graders used in grouper larviculture. J. Fish Soc. Taiwan 31: 67-71.

Kennedy SB, Tucker JW, Neidic CL, Vermeer GK, Cooper VR, Jarrell JL, Sennett DG (1998). Bacterial management strategies for stock enhancement of warm water marine fish: a case study with common snook (*Centropomus undecimalis*). Bull. Mar. Sci. 62: 573-588.

Martin-Antonio B, Manchado M, Infante C, Zerolo R, Labella A, Alonso C, Borrego JJ (2007). Intestinal microbiota variation in Senegalese sole (*Solea senegalensis*) under different feeding regimes. Aquac. Res. 38: 1213-1222.

Mazmanian SK, Round JL, Kasper DL (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453: 620-624.

Munro PO, Barbour A, Birkbeck TH (1994). Comparison of the gut bacterial flora of start-feeding larval turbot reared under different

conditions. J. Appl. Microbiol. 77: 560-566.

Muroga K, Higashi M, Keitoku H (1987). The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at larval and juvenile stages. Aquac. 65: 79-88.

Olafsen JA (2001). Interactions between fish larvae and bacteria in marine aquaculture. Aquac. 200: 223-247.

Olsson JC, Westerdahl A, Conway PL, Kjelleberg S (1992). Intestinal colonization potential of Turbot (*Scophthalmus maximus*) and Dab (*Limanda limanda*) -associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 58, 551-556.

Ringø E, Myklebust R, Mayhew TM, Olsen RE (2007). Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. Aquac. 268: 251-264.

Ringø E, Olsen RE, Øverli Ø, Løvik F (1997). Effect of dominance hierarchy formation on aerobic microbiota associated with epithelial mucosa of subordinate and dominant individuals of Arctic charr, *Salvelinus alpinus* (L.). Aquac. Res. 28: 901-904.

Ringø E, Sinclair PD, Birkbeck H, Barbour A (1992). Production of eicosapentaenoic acid (20:5 n-3) by *Vibrio pelagius* isolated from turbot (*Scophthalmus maximus* (L)) larvae. Appl. Environ. Microbiol. 58: 3777-3778.

Ringø E, Sperstad S, Myklebust R, Mayhew TM, Olsen RE (2006a). The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.). Aquac. Res. 37: 891-897.

Ringø E, Sperstad S, Myklebust R, Refstie S, Krogdahl A (2006b). Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.) The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. Aquac. 261: 829-841.

Romero J, Navarrete P (2006). 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). Microb. Ecol. 51: 422-430.

Skrodenyt -Arba iauskien V (2007). Enzymatic activity of intestinal bacteria in roach *Rutilus rutilus* L. Fish. Sci. 73: 964-966.

Spanggaard B, Huber I, Nielsen J, Nielsen T, Appel KF, Gram L (2000). The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquac. 182: 1-15.

Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen T, Slierendrecht WJ, Gram L (2001). The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Envion. Microbiol. 3: 755-765.

Sugita H, Hirose Y, Matsuo N, Deguchi Y (1998). Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. Aquac. 165: 269-280.

Sugita H, Ito Y (2006). Identification of intestinal bacteria from Japanese flounder (*Paralichthys olivaceus*) and their ability to digest chitin. Lett. Appl. Microbiol. 43: 336-342.

Sugita H, Takahashi J, Miyajima C, Deguchi Y (1991). Vitamin B12-producing ability of the intestinal microflora of Rainbow trout (*Oncorhynchus mykiss*). Agric. Bio. Chem. 92: 267-276.

Sugita H, Okano R, Suzuki Y, Iwai D, Mizukami M, Akiyama N, Matsuura S (2002). Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. Fish Sci. 68: 1004-1011.

Sumpter JP (1992). Control of growth of rainbow trout (*Oncorhynchus mykiss*). Aquac. 100: 299–320.

Sun YZ, Mao SY, Yao W, Zhu WY. (2008). DGGE and 16S rDNA analysis reveals a highly diverse and rapidly colonizing bacterial community on different substrates in the rumen of goats. Animal 2: 391-398.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids. Res. 25: 4876- 4882.

Velmurugan S, Rajagopal S (2009). Beneficial uses of probiotics in mass scale production of marine ornamental fish. Afr. J. Microbial. Res., 3: 185-190.

Verner-Jeffreys DW, Shields RJ, Bricknell IR, Birkbeck TH (2003). Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. Aquac. 219: 21-42.

Verschuere L, Rombout G, Sorgeloos P, Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64: 655-671.

Vine NG, Leukes WD, Kaiser H (2006). Probiotics in marine larviculture. FEMS. Microbiol. Rev. 30: 404-427.

Yeh SL, Kuo CM, Ting YY, Chang CF (2003). Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. Comp. Biochem. Physiol. Part C. Toxicol. Pharmacol. 135C: 375-382.