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Probiotic *Psychrobacter* sp. improved the autochthonous microbial diversity along the gastrointestinal tract of grouper *Epinephelus coioides*

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Abstract

The effect of dietary administration of probiotic *Psychrobacter* sp. SE6 for 60 days on the autochthonous microbiota in the foregut, midgut and hindgut of juvenile grouper *Epinephelus coioides* was assessed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). A complex and generally similar bacterial composition was present along the gastrointestinal (GI) tract of fish fed the probiotic or control diet. However, samples collected from the probiotic group and control group showed different DGGE patterns. The similarity dendrogram demonstrated that all nine samples from the control group were closely related and distinctly different to the probiotic samples. The total number of bands and Shannon index of the foregut, midgut and hindgut samples in the probiotic group were significantly higher than those in the control group, suggesting probiotic *Psychrobacter* sp. improved the autochthonous microbial diversity along the GI tract of *E. coioides*. Some potentially beneficial and uncultured bacteria were stimulated, while some potentially harmful species, such as *Staphylococcus saprophyticus*, were suppressed. Sequence analysis showed that the majority of bacterial sequences (48.0%) in this study were highly similar to unidentified clones, suggesting a considerable proportion of unknown bacteria in the gut of *E. coioides*.

Keywords: Gut microbiota; Probiotic; *Psychrobacter* sp; *Epinephelus coioides*; DGGE

Introduction

The increased intensification of aquaculture has led to a high number of disease outbreaks with an increasing range of pathogens. Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance, and also due to concerns over environmental impacts. Therefore, the use of probiotics has been suggested to be an alternative method for the prevention and control of various diseases in aquaculture [1-3]. Recently, the potential of using probiotic *Lactobacillus plantarum* [4] and *Bacillus* sp. [5] for disease control, immune stimulation and growth promotion have been demonstrated in grouper *Epinephelus* spp., one of the most important mariculture fish species in China and Southeast Asian countries [4]. However, the mechanisms behind these benefits are not well understood. Recently, it is suggested that a clear understanding on the effect of probiotics on the autochthonous gut microbiota is integral to illustrate the mechanisms responsible for probiotic benefits [3].

The gastrointestinal (GI) tract of fish harbours a complex microbial community, including two distinct groups, i.e. allochthonous (exogenous) and autochthonous (indigenous) [1]. Autochthonous organisms are reported to play important roles within the GI tract, including the capacity to contribute to the development/maturation of the gut and immune system [1,6,7], and provide resistance to infectious pathogens [8,9]. The autochthonous microbiota has also been reported to inhibit the colonization of introduced bacteria by mechanisms including space occupation, competition for substances and receptors at mucosal surfaces, and secretion of inhibitory substance [2]. However, little information is available on the impact of probiotics administration on the gut autochthonous microbiota of fish [2,3]. Moreover, previous gut microbial studies often have used cultivation-based techniques, which obviously only allow the investigation of culturable bacteria, while the effect on non-culturable bacteria, which may account for the majority of the bacterial population in the gut of fish, remained largely unclear [2,3,10]. As an alternative, genetic fingerprint methods based on polymerase chain reaction (PCR) amplification of 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) have been successfully applied in gut microbiota study [3,10-15].

Recently, we compared the gut microbiota of fast and slow growing grouper *Epinephelus coioides*. The results showed that *Psychrobacter* sp. SE6 dominated in the gut of fast growing fish, but was absent in the gut of slow growing fish [16]. *In vitro* study showed that this strain exhibited antagonistic activity against several fish pathogens [16]. Subsequently, a 60 days of feeding trial confirmed that it could improve the feed utilization and immune responses of juvenile grouper *E. coioides* [17]. In the present study, PCR-DGGE with subsequent sequence analysis was used to assess the effect of dietary administration of probiotic *Psychrobacter* sp. SE6 on the autochthonous microbiota along the GI tract of grouper *E. coioides*.

Materials and Methods

Bacterial strain

Psychrobacter sp. SE6 was isolated from the whole intestine of juvenile grouper *E. coioides* and identified based on physiological and biochemical tests, such as cell shape, pigmentation, Gram stain, catalase test, utilization of Simmon Citrate, sugar fermentation and so on, and this strain was characterized further by 16S rRNA gene sequencing

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(GenBank accession number: EU520334) [16]. The strain was cultured and prepared as described previously [16]. Briefly, 50 µl of *Psychrobacter* sp. storage solution was inoculated in 20 ml nutrient broth. After 24 h of incubation, the cells were harvested and re-suspended in PBS for addition to the basal diet. The number of bacteria in the suspension was 1.0×10^{10} cells ml^{-1}, which was determined by plate counting on tryptone soya agar (TSA) at 28°C for 48 h.

Diet preparation and feeding trial

The control diet was formulated using the ingredients as showed in Table 1. The probiotic diet was prepared by gently spraying the required amount of bacterial suspension on the control diet (10 ml bacterial suspension per kg diet) and mixing it part-by-part in a three dimensions drum mixer (SYH-100, Punaier Drying Equipment Co., Ltd, Changzhou, China) to obtain a final probiont concentration of 1.0×10^{8} cells g^{-1}. Dietary ingredients of the respective probiotic and control diets were mixed with required amount of water and then cold press extruded (CD4XITS extruder, South China University of Technology, Guangzhou, China) to produce 5 mm pellets, which were dried for 3 days at room temperature (20-25°C) and packed in sterile polypropylene containers and stored at 4°C. The counts of probiotic *Psychrobacter* sp. in the diets were determined by spread plating on TSA as described by [17].

Juvenile grouper *E. coioides* were obtained from a local commercial farm and transported to the Aquaculture Research Aquarium, University of Jimei, China. Fish were fed the control diet and acclimated for 4 weeks before the beginning of the trial. The feeding experiment was conducted in six 180-l seawater fibreglass tanks, each connected to an open circulating system (35 g l^{-1} salinity, at 28 ± 2°C). Each tank was randomly stocked with 14 fish (45.02±0.18 g) and each treatment was conducted in triplicate. Fish were fed the control diet or probiotic diet. The fed level was 3% biomass per day provided in equal rations at 09:00 and 17:00 h for 60 days.

At the end of the trial (day 60), three individual fish from each treatment were randomly collected and the GI tract of each fish was sampled and divided into foregut, midgut and hindgut as previously described [18]. The foregut refers to the stomach, the midgut includes the pyloric caeca and proximal intestine as fish pyloric caeca has similar functions as proximal intestine [19], and the hindgut refers to distal portion of the intestine. Each section was aseptically excised and the digesta was removed as described in [20]. Each GI section was homogenized using a glass homogenizer and stored at −80 °C until further analysis. In the present study, three individual fish in each group (fish C1, C2 and C3 in the control group, fish P1, P2 and P3 in the probiotic group) were investigated as previous studies have demonstrated inter-fish variation in the gut microbiota [20,21].

Ingredients	Control	Probiotic
Fish meal	600	600
Soybean meal	160	160
Shrimp meal	20	20
Wheat flour	140	140
Fish oil	40	40
Soybean phospholipids	20	20
Vitamin mixture*	10	10
Mineral mixture†	10	10
Psychrobacter sp.	0	1.0×10^{8}

*Vitamin and †mineral mixture for *Epinephelus coioides* provided by Haikang Feed Company, Zhaoan, Zhangzhou, China.

Table 1: Composition of the basal diet (g kg^{-1}) and probiotic level (cells g^{-1}) for *Epinephelus coioides*.

DNA extraction, PCR amplification and DGGE analysis

Total DNA was extracted from the homogenates of GI sections as described by [22]. Primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3' with a GC clamp CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG at the 5' end) and 519r (5'-ATTACCGCGGCTGCTGG-3') were used to amplify the V3 region of the bacterial 16S rRNA gene [23]. A touchdown PCR [12] was performed for all samples to reduce nonspecific priming by using a MJ Mini thermocycler (Bio-Rad, Hercules, California, USA).

PCR products of the V3 region of 16S rRNA gene from the gut samples were used for sequence-specific separation by DGGE [22], using a Dcode TM system (Bio-Rad, Hercules, CA, USA). DGGE was performed in 8% polyacrylamide gels containing 37.5:1 acrylamide-biacrylamide and a denaturing gradient of 35-50% of urea and formamide. All PCR products (10 µl for each sample) were loaded on the same gel. The electrophoresis was initiated by pre-running for 10 min at 200 V, and subsequently ran at a fixed voltage of 85 V for 12 h at 60°C. After completion of electrophoresis, the gel was stained in 0.2% $AgNO_3$ solution for 10 min and then visualized in a visualization solution (trace NaBH4 in 1.5% NaOH), and scanned using GS 800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA).

Analysis of DGGE patterns

DGGE patterns were analyzed as described in [22] using software of GelCompar® (Applied Maths, Austin, TX, USA). Levels of similarity between fingerprints were calculated according to the Dice similarity coefficient (S_D) as previously described [24]. The unweighted pair group method with arithmetic averages (UPGMA) was used to create a dendrogram representing the similarity of the microbial profiles from the DGGE fingerprints [22]. In order to determine the structural diversity of the microbial community corresponding to the DGGE banding pattern, two indices were calculated: (1) the species richness (R) was calculated based on the total number of bands. (2) the Shannon index (*H'*) which reflects the diversity of the whole microbial community. These 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 Sciences (SPSS), release 14.0 (SPSS, Inc., Chicago, IL). Significant differences were declared at $P\leq0.05$.

Sequencing of DGGE bands

Bands of interest were excised for sequence analysis as previously described [22]. Briefly, DNA was eluted from excised bands in 50 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at 4 °C overnight. PCR was performed using 2µl of the elution as template to amplify the V3 region of 16S rRNA gene with primers 338f and 519r [23]. The PCR products were cleaned using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and sequenced by Invitrogen Biotechnology Co., Ltd (Shanghai, China). The sequences were checked for chimeric constructs by using the CHECK CHIMERA program of the ribosomal database project (RDP) [25]. The resulting sequences (~200 bp) were compared with the sequences from the National Center for Biotechnology Information (NCBI) using the BLAST sequence algorithm to retrieve the closest known alignment identities. The sequences reported in this study have been deposited in the GenBank database under the following accession numbers: lcl951, lcl9953, lcl16591, lcl17197, lcl22649, lcl25487, lcl25609, lcl28133, lcl28577, lcl28947, lcl29719, lcl31463, lcl34599, lcl36143, lcl38265, lcl43263,

lcl44189, lcl52867, lcl54423, lcl54911, lcl55019, lcl56485, lcl58261, lcl58447 and lcl63159.

Results

Gut microbiota analysis by DGGE

The PCR-DGGE techniques were employed to evaluate the autochthonous bacterial diversity of the foregut, midgut and hindgut samples from fish fed the probiotic diet and control diet (Figure 1). In general, samples collected from probiotic group had similar DGGE patterns, with 20 to 26 bands to each sample, while samples collected from the control group also showed similar DGGE patterns, with 14 to 19 bands to each sample (Figure 1). This suggested that the bacterial community was generally similar among the three GI sections of fish fed the probiotic diet or control diet (Figure 2), and the probiotic diet might increased the bacterial diversity along the GI tract of *E. coioides*.

The similarty dendrogram showed that all nine samples from the control group were closely related, with a high similarity index (62.5%), which was distinctly different to the probiotic samples (Figure 2). The relatively low similarities between the probiotic and control samples in the similarity dendrogram confirmed the visual differences in DGGE profiles. The total number of bands (R) and the Shannon index (*H'*) of the foregut, midgut and hindgut samples in the probiotic group were significantly higher than those in the control group (Figure 3a, 3b), which further confirmed that the probiotic diet increased the autochthonous microbial diversity along the GI tract of *E. coioides*.

Sequences from bands in DGGE gel

A total of 27 bands (band 1-27) were excised from the DGGE gel, and 25 bands were successfully sequenced (Figure 1) and the BLAST results were present in Table 2. The 25 identified bacteria were closely related to one of the following four groups: Proteobacteria (32.0% of the total), Actinobacteria (12.0%), Firmicutes (8.0%) and unclassified bacteria (48.0%). Twelve common bacteria (corresponding to band 1-12) were present in all samples, including *Pseudomonas* sp. CB10-like,

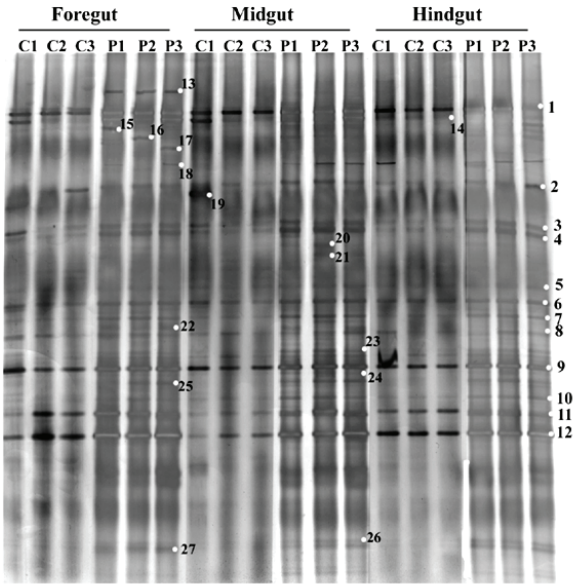

Figure 1: DGGE profiles of the foregut, midgut and hindgut samples in *Epinephelus coioides* fed the control diet and probiotic diet for 60 days. C1, C2 and C3 represent the three fish fed the control diet, while P1, P2 and P3 represent the three fish fed the probiotic diet. Band 1-12 are common bands to all samples, band 14 and 19 are present only in the control group, while band 13, 15-18, 20-27 only in the probiotic group. Those bands are sequenced and described in Table 2.

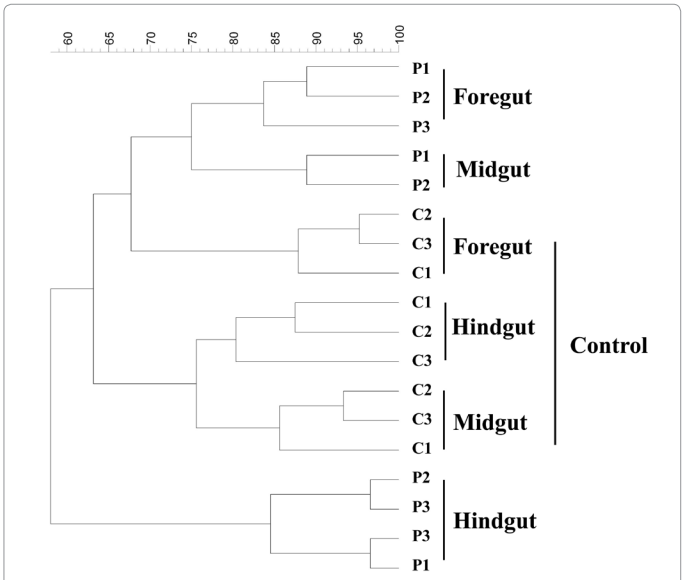

Figure 2: Unweighted pair group clustering dendrogram of DGGE profiles using arithmetic average linkage. C1, C2 and C3 represent the three fish fed the control diet, while P1, P2 and P3 represent the three fish fed the probiotic diet.

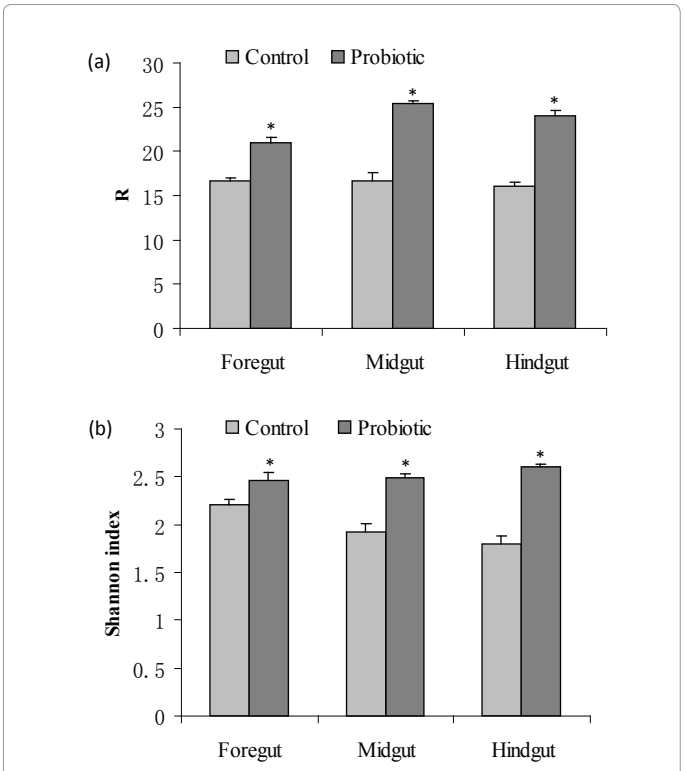

Figure 3: Analysis of DGGE banding patterns from samples of the foregut, midgut and hindgut in *Epinephelus coioides* fed the control diet and probiotic diet for 60 days. a. Number of DGGE bands (*R*). b. Shannon index (*H'*). *significant difference between the fish fed the probiotic diet and control diet (*P≤0.05*).

Nitratireductor sp. YCSC5-like, *Methylobacterium hispanicum* strain-like, *Microbacterium* sp. YACS1-like, *Dietzia* sp. N11-like bacterium and seven uncultured bacterium clone-like bacteria.

Twelve bands (band 13, 15-17 and 20-27) were present only in the probiotic group (Figure 1). Band 13, 15-17 and 22 were observed only in the foregut, band 13 and 22 observed in three fish and showed 94% and 99% similarity to *Micrococcus luteus* and uncultured beta proteobacterium isolate DGGE gel band YL6, respectively, while band 15, 16 and 17 observed in one fish (fish P1, P2 and P3, respectively) and were most closely related to uncultured bacterium. Band 20 and 21 were present in the midgut and most closely related to *Alcanivorax dieselolei* strain Qtet3 and uncultured alpha proteobacterium clone A22YB05RM, respectively. Band 23-27 were observed in all the probiotic samples, band 23, 24 and 25 were most closely related to uncultured bacterium.

On the contrary, band 19 appeared only in midgut of fish C1 fed the control diet and showed 98% similarity to *Bacillus* sp. JZDN5. Band 14 presented in all the probiotic samples, but missed in the hindgut samples of the control group and showed 99% similarity to *Staphylococcus saprophyticus*.

Discussion

The effect of dietary administration of probiotic *Psychrobacter* sp. SE6 on the autochthonous microbiota along the GI tract of *E. coioides* was evaluated for the first time using PCR-DGGE with subsequent sequencing analysis. Our data demonstrated that probiotic *Psychrobacter* sp. significantly increased the autochthonous microbial diversity (visible band number and Shannon diversity index) of *E. coioides*. In line with our finding, dietary administration of fresh or lyophilized probiotic *Shewanella putrefaciens* Pdp11 for 60 days exerted an important influence on intestinal bacterial DGGE profiles and yielded a faster stabilization of the bacterial community in flat fish *Solea senegalensis* [14]. On the contrary, dietary supplementation with *Pediococcus acidilactici* for 32 days significantly reduced the microbial diversity and richness in the intestine of red tilapia (*Oreochromis niloticus*) [26]. Therefore, different probiotic strains may exert different effect on the gut microbiota of fish. However, direct comparisons between these studies are also difficult because these strains are functionally different and the results may be affected by genetic, nutritional and environmental factors.

Phylogenic group	Band no.	Closest relative	Similarity (%)	Accession no.
Proteobacteria	1	*Pseudomonas* sp. CB10	99%	lcl29719
	9	*Nitratireductor* sp. YCSC5	100%	lcl22649
	10	*Methylobacterium hispanicum* strain	94%	lcl28133
	18	Uncultured alpha proteobacterium	94%	lcl38265
	20	Uncultured alpha proteobacterium clone A22YB05RM	92%	lcl31463
	21	*Alcanivorax dieselolei* strain Qtet3	88%	lcl25609
	22	Uncultured beta proteobacterium isolate DGGE gel band YL6	96%	lcl54423
	23	Uncultured alpha proteobacterium	100%	lcl58447
Firmicutes	14	*Staphylococcus saprophyticus*	99%	lcl25487
	19	*Bacillus* sp. JZDN5	98%	lcl28577
Actinobacteria	11	*Microbacterium* sp. YACS1	99%	lcl58261
	12	*Dietzia* sp. N11	98%	lcl16591
	13	*Micrococcus luteus*	94%	lcl63159
Unclassified bacteria	2	Uncultured bacterium clone HKTUI136	98%	lcl54911
	3	Uncultured bacterium clone FATNRJA N079	99%	lcl43263
	4	Uncultured bacterium clone 16saw7707	98%	lcl951
	5	Uncultured bacterium partial 16S rRNA gene, clone 9-C02	97%	lcl44189
	6	Uncultured bacterium clone CX-1	99%	lcl28947
	7	Uncultured bacterium clone D3T-094	95%	lcl56485
	8	Uncultured bacterium clone S2-8B	99%	lcl52867
	15	Uncultured bacterium isolate DGGE band 34	94%	lcl55019
	16	Uncultured bacterium clone pl14H11	95%	lcl36143
	17	Uncultured bacterium clone STSAO- B8	86%	lcl34599
	24	Uncultured bacterium isolate DGGE gel band L4B5	100%	lcl9953
	25	Uncultured bacterium clone C61	96%	lcl17197

Table 2: Summary of BLAST search data arising from the bands in the DGGE gel (Figure 1) of gastrointestinal samples in *Epinephelus coioides* fed the probiotic diet and control diet for 60 days.

In this study, probiotic *Psychrobacter* sp. improved the autochthonous microbial diversity by stimulating the growth of many bacteria, including a *Micrococcus luteus*-like bacterium, an *Alcanivorax dieselolei*-like bacterium and several uncultured bacteria. *Micrococcus* spp. has been isolated from the GI tract of several coastal fish [27] and gonads of Nile tilapia *O. niloticus* [28]. Dietary administration of *M. luteus* improved the growth and health of *O. niloticus* [28]. Furthermore, *M. luteus* is currently marketed as probiotics for aquaculture in India (Prowins Biotech Private Ltd., India). Therefore, we speculated that this stimulation of *M. luteus*-like bacterium by probiotic *Psychrobacter* sp. may be beneficial to the host. Another stimulated bacterium, *A. dieselolei*-like bacterium, is belonging to γ-Proteobacteria, has been isolated from sea water and deep-sea sediment [29-30]. Nakano et al. [30] found that *A. dieselolei* N1203 derived from marine sediments is a novel type of denitrifying bacterium. To our knowledge, however, *A. dieselolei*-like bacterium in the gut of fish has been identified for the first time in the present study, and its role in the gut is not clear and further study is needed.

On the other hand, probiotic *Psychrobacter* sp. depressed *Staphylococcus saprophyticus*-like bacterium to an undetectable level in the hindgut of *E. coioides*. Although there are no reports that *S. saprophyticus* caused diseases in fish, we speculate that *S. saprophyticus* may be a potentially harmful bacterium as this strain has been implicated in human urinary tract infections [31] and members of the genus *Staphylococcus* have been suggested as pathogens for marine and freshwater fish [32,33]. Therefore, probiotic *Psychrobacter* sp. seemed to exert a competitive effect on potentially pathogenic bacteria in the GI tract of *E. coioides*. Indeed, as one of the most dominant bacteria in the gut of *E. coioides*, *Psychrobacter* sp. has previously showed an in vitro antagonistic effect to a number of pathogenic species, such as *S. aureus*, *Vibrio harveyi*, *Vibrio metschnikovi* and *Vibrio alginolyticus* [16]. It is not surprising that dietary administration of *Psychrobacter* sp. SE6 inhibited the growth of some pathogenic bacteria and therefore benefited the growth of many potentially beneficial or neutral bacteria in the gut. Consequently, the gut microbial diversity increased following the administration of probiotic *Psychrobacter* sp. and this may be beneficial to the growth and health of *E. coioides* [17].

Culture-based studies have demonstrated that different autochthonous microbial communities were present in different gut sections of fish, like Atlantic salmon *S. salar* L. [34] and Atlantic cod *Gadus morhua* L. [20]. However, similar DGGE patterns were observed in the anterior mucosa (AM) and the posterior mucosa (PM) samples of rainbow trout *O. mykiss* (Walbaum) [35], and similar bacterial composition in the stomach, pyloric caeca and intestine of juvenile Atlantic salmon *S. salar* L. was demonstrated by using different fingerprinting techniques such as temporal temperature gradient gel electrophoresis (TTGE) and restriction fragment length polymorphism (RFLP) [13]. In line with those previous molecular findings, a generally similar bacterial composition along the digestive tract was observed in *E. coioides* fed the probiotic diet or control diet in this study and our previous study [36]. On the contrary, Zhou et al. [10] observed different bacterial composition in the stomach, pyloric caeca and intestine of grouper *E. awoara* using PCR-DGGE technique. Therefore, the microbiota along the digestive tract of fish need further study.

Previous DGGE studies have demonstrated that high levels of unknown species are present in the gut of fish, such as rainbow trout *O. mykiss* (Walbaum) [12], hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂) [21], grouper *E. awoara* [10] and flat fish *S. senegalensis* [14]. In line with those previous studies, the majority of bacterial sequences (48.0%) in this study were highly similar to unidentified clones, suggesting a considerable proportion of unknown bacteria in the gut of *E. coioides*. A possible reason is that such organisms are fastidious and difficult to culture, and are thus not well documented. Therefore, it is suggested that culture-independent approaches may lead to the discovery of novel or unidentified bacteria and the gut microbiota of fish are not as simple as earlier believed [10]. Future probiotic studies should pay more attention to the gut microbiota of fish and evaluate it by molecular techniques, such as PCR-DGGE, quantitative real-time PCR, ribosomal intergenic spacer analysis (RISA) and RFLP. These findings will broaden our understanding of the probiotic effects at the gut level, which is integral to understand the mechanisms which underpin host benefits.

In summary, dietary supplementation of probiotic *Psychrobacter* sp. SE6 improved the indigenous microbial diversity along the GI tract of *E. coioides* by depressing some potentially harmful species and stimulating some potentially beneficial and uncultured bacteria.

Acknowledgments

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