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Probiotic *Bacillus pumilus* SE5 shapes the intestinal microbiota and mucosal immunity in grouper *Epinephelus coioides*

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ABSTRACT: The health benefits of probiotics are thought to occur, at least in part, through an improved intestinal microbial balance in fish, although the molecular mechanisms whereby probiotics modulate the intestinal microbiota by means of activation of mucosal immunity are rarely explored. In this study, the effects of viable and heat-inactivated probiotic *Bacillus pumilus* SE5 on the intestinal dominant microbial community and mucosal immune gene expression were evaluated. The fish were fed for 60 d with 3 different diets: control (without probiotic), and diets T1 and T2 supplemented with 1.0×10^8 cells g^{-1} viable and heat-inactivated *B. pumilus* SE5, respectively. Upregulated expression of TLR1, TLR2 and IL-8, but not MyD88 was observed in fish fed the viable probiotic, while elevated expression of TLR2, IL-8 and TGF-β1, but not MyD88 was observed in fish fed the heat-inactivated *B. pumilus* SE5. The induced activation of intestinal mucosal immunity, especially the enhanced expression of antibacterial epinecidin-1, was consistent with the microbial data showing that several potentially pathogenic bacterial species such as *Psychroserpens burtonensis* and *Pantoea agglomerans* were suppressed by both the viable and heat-inactivated probiotic *B. pumilus* SE5. These results lay the foundation for future studies on the molecular interactions between probiotics, intestinal microbiota and mucosal immunity in fish.

KEY WORDS: *Bacillus pumilus* · Intestinal microbiota · Mucosal immunity · Toll-like receptor · *Epinephelus coioides*

INTRODUCTION

The increased intensification of marine fish aquaculture has led to a high number of disease outbreaks with an increasing range of bacterial 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 (Merrifield et al. 2010). Therefore, the use of probiotics has been suggested as an alternative method for the prevention and control of various diseases in aquaculture (Merrifield et al. 2010, Ringø et al. 2010). Recently, the potential of using probiotics 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 (Son et al. 2009, Sun et al. 2010, 2011, Harikrishnan et al. 2010). Our research group observed that *Bacillus pumilus* SE5 was a dominant bacterium in the gut of fast growing grouper *Epinephelus coioides* and exhibited *in vitro* antagonistic activity against several fish pathogens (Sun et al. 2009). Subsequently, an *in vivo* study confirmed that viable *B. pumilus* SE5 could improve the feed utilization and immune responses

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(Sun et al. 2010) and modulate the gut microbiota of juvenile *E. coioides* (Sun et al. 2011).

Although the mechanisms are not well understood, Merrifield et al. (2010) suggests that the benefits of probiotics in fish are achieved, at least in part, via improving the host intestinal microbial balance. However, little information is available about the molecular mechanisms by which probiotics modulate the gut microbiota of fish through the activation of mucosal immunity. In homothermic animals, it is generally accepted that toll-like receptors (TLRs) of the intestinal mucosal immune system can recognize microbial-associated molecular patterns (MAMPs) shed by commensal microbes/probiotics and activate signalling cascades that finely tune the production of cytokines and antimicrobial proteins depending on the signals delivered by the microbes, which helps to establish a balanced microbial community (Abreu 2010, Sánchez de Medina et al. 2013). It has been proposed that the fish mucosal immune system may have a similar response to probiotics, but little information is available (Pérez et al. 2010, Rombout et al. 2011).

Viable probiotics modulating the intestinal microbiota of fish has been extensively reported (Tapia-Paniagua et al. 2010, Ferguson et al. 2010, Sun et al. 2011, Liu et al. 2013). Interestingly, several recently published studies have demonstrated that certain dead probiotics can modulate the gut microbial community of fish (Hoseinifar et al. 2011, Mohapatra et al. 2012) and improve gut mucosal immunity (Salinas et al. 2008, Pan et al. 2008, Forberg et al. 2012). As the dead probiotics could not compete for nutritional substances, nor secrete inhibitory substances in the gut, the most likely mechanism whereby the dead probiotics modulate gut microbiota is by activating the mucosal immune system of host. Therefore, dead probiotics could be good research subjects to study the molecular mechanisms in interactions between probiotics, gut microbiota and mucosal immunity in fish. In the present study, therefore, the effects of viable and heat-inactivated probiotic *B. pumilus* SE5 on the intestinal microbiota and mucosal immune gene expression in *E. coioides* were evaluated.

MATERIALS AND METHODS

Probiotic strain and diet preparation

Probiotic *Bacillus pumilus* SE5 was isolated from the gut of juvenile grouper *Epinephelus coioides* (Sun et al. 2009), and cultured and prepared as described previously (Sun et al. 2009, 2010). After incubation, the cells were harvested and resuspended in phosphate-buffered saline. 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. Part of the live bacterial suspension was heat-inactivated in a water bath at 95°C for 60 min, and non-viability was checked by plating on TSA.

In our previous studies, probiotic *B. pumilus* SE5 in a generally accepted dose (1.0×10^8 CFU g^{-1}) has been confirmed to be effective in improving the feed efficiency and immune response (Sun et al. 2010), and modulating the gut microbiota of grouper *E. coioides* (Sun et al. 2011). In the present study, therefore, the same dose (1.0×10^8 CFU g^{-1}) was used to assess the effect of viable and heat-inactivated SE5 on the intestinal microbiota and mucosal immune gene expression in *E. coioides*. The basal diet was formulated as described in Sun et al. (2011). Probiotic diets T1 and T2 were prepared by gently spraying the required amount of viable and heat-inactivated bacterial suspensions, respectively, on the control diet and mixing it part-by-part in a 3-dimensional drum mixer (SYH-100, Punaier Drying Equipment) to obtain a final probiotic concentration of 1.0×10^8 cells g^{-1}. Dietary ingredients of the probiotic and control diets were mixed with required amount of water and then cold press extruded (CD4XITS extruder, South China University of Technology) to produce 5 mm pellets. The counts of SE5 in the T1 and T2 diets after extrusion were determined by spread plating on TSA as described in Sun et al. (2011); a high probiotic level (0.96×10^8 CFU g^{-1}) was observed in the T1 diet, while no viable SE5 was observed in the T2 diet.

Experimental design

The animal trial in this study followed the protocols approved by animal care and use committee of Jimei University, China. Juvenile grouper *E. coioides* were obtained from a local commercial farm and transported to the Aquaculture Research Aquarium, Jimei University. The feeding experiment was conducted in nine 180 l seawater fiberglass tanks, each connected to an open circulating system (30 g l^{-1} salinity, at 26 ± 2°C, mean ± SE). Each tank was randomly stocked with 25 fish (14.57 ± 0.05 g) and each treatment was conducted in triplicate. The fish were fed the control diet, viable probiotic containing diet (treatment T1) and heat-inactivated probiotic containing diet (treatment T2), respectively. The feeding level was 3% biomass d^{-1} provided in equal rations at 09:00 and

17:00 h for 60 d. At the end of the trial (Day 60), 3 fish were taken randomly from each tank, and thus a total of 9 fish were collected per treatment. The intestine of each fish was aseptically excised and the digesta was removed under sterile conditions as described by Sun et al. (2011). Three intestinal samples per treatment (1 sample from each tank) were kept in Eppendorf tubes at –80°C for DNA extraction and microbial analysis, and the other 6 intestinal samples per treatment (2 samples from each tank) were stored at –80°C in TRIzol reagent (Invitrogen) for RNA extraction and immune gene analysis. It should be noted that one of the experimental aims, not presented in this study, was to evaluate the effect of probiotic treatment on growth performance. This accounts for the disparity in the number of fish used and number of fish sampled for microbiota and immunological analyses.

Bacterial community analysis

The dominant bacterial communities of 3 individual fish in each group (fish C1, C2 and C3 in the control group, fish V1, V2 and V3 in the viable probiotic group and fish D1, D2 and D3 in the heat-inactivated probiotic group) were analyzed. Total DNA extraction, PCR, denaturing gradient gel electrophoresis (DGGE) and sequencing were conducted as described in Sun et al. (2011). DGGE fingerprints were analyzed using Quantity One v.4.6.3 analyses software (Bio-Rad Laboratories). Levels of similarity between fingerprints were calculated according to the Dice similarity coefficient and the unweighted pair group method with arithmetic averages (UPGMA) was used to create a dendrogram. The resulting sequences 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: KC991203, KC991208, KC991211, KC991212, KC991215, KC991217, KC991221, KC991222, KC991223, KC991225, KC991229, KC991233 and KC991234.

Immune genes expression analysis

Intestinal tissues were homogenized and total RNA was extracted from each homogenized tissue sample by the TRIzol extraction method according to the manufacturer (Invitrogen). Quantification was carried out with a NanoDrop 1000 (Thermo Scientific) and the quality of the RNA was checked with a Bioanalyzer (Agilent technologies). To remove DNA, 1.5 µg RNA were treated with 2 µl DNase, 2 µl DNase buffer and appropriate RNase free water (Sigma) in a final volume of 11 µl according to the protocol of the manufacturer (Promega). Subsequently, first-strand cDNA was synthesized using a TIANscript RT Kit (Tiangen). Briefly, the DNase treated RNA was mixed with 1 µl random primer (Sangon). The mix was first incubated at 70°C for 5 min and then rapidly cooled on ice for 2 min. Next, the solution containing 1 µl Ribolock RNase Inhibitor, 1 µl Quant reverse transcriptase, 4 µl 10× Reaction buffer, 1 µl dNTP mix (10 mM each nucleoside) and 1 µl RNase free water in a final volume of 20 µl was incubated at 37°C for 1 h.

The mucosal immune genes, such as TLR1, TLR2, TLR5, MyD88, IL-1β, IL-8, TGF-β1, epinecidin-1 and IgM, were determined using RT-qPCR with specific primers as previously reported (Table 1). β-actin was selected as the housekeeping gene for data normalization, as it has been extensively reported to be a good housekeeping gene for *E. coioides* gene expression studies (Xu et al. 2010, Wei et al. 2011). The RT-qPCR was performed with the SYBR Green Realtime PCR Master Mix (Toyobo) in an ABI 7500 real-time PCR Detection system (Applied Biosystems). The total volume of the PCR reactions was 20 µl and consisted of 10 µl 2× SYBR GreenIRealtime PCR Master Mix, 0.5 µl primer of each, 2 µl cDNA, and 7 µl deionized H_2O. The cycling conditions were as follows: 95°C for 1 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. All RT-qPCRs were performed at least 3 times.

Statistical analysis

The data of expression of immune genes from 6 fish are presented as fold increases (mean ± SE). Data were examined by 1-way analysis of variance (ANOVA). When ANOVA identified differences among groups, a multiple comparison (Duncan's) test was conducted to examine significant differences among treatments using Statistical Package for Social Science (SPSS), release 14.0. Significant differences were declared at $p \leq 0.05$.

RESULTS

Bacterial community

The autochthonous bacterial compositions of intestinal samples from 3 fish fed either the control

Table 1. Real-time PCR primers used for immune genes of the grouper *Epinephelus coioides*

Gene	Nucleotide sequence (5′-3′)	Accession no.	Reference
β-actin	Fw: GAC ATC AAG GAG AAG CTG TG Rev: TGC TGT TGT AGG TGG TCT CGT	AY510710	Wei et al. (2011)
TLR1	Fw: CCA GGG TCG CAG AGT CCT ATC Rev: GCC AGC CAA GTT CAG TTT CGT	HM357229	Wei et al. (2011)
TLR2	Fw: AGG GTT CAG AAG GGT TGC TAT Rev: CAG GAA GGA AGT CCC GTT TGT	HM357230	Wei et al. (2011)
TLR5	Fw: CTG ACC CTG ATG CTT TTC G Rev: GCT ACT TTA CTG CTG TGT G	GH612592	Xu et al. (2010)
MyD88	Fw: AGC TGG AGC AGA CGG AGT G Rev: GAG GCT GAG AGC AAA CTT GGT C	GQ202584	Wei et al. (2011)
IL-1β	Fw: AGG ATG CCT GAG GGA CTG Rev: GGT AAT CGT CTC CAG ATG TAA	EF582837	Lu et al. (2008)
IL-8	Fw: GCA AGC TTG GCG TTT TTT GGT GTT GGC CAT Rev: CTG GGT ACC ATG AGC AGC AGA GTC ATT GTC	FJ913064	Hu et al. (2010)
TGF β1	Fw: CAC CTA CAT CTG GAA TGC TGA AAA C Rev: CTG CTC CAC CTT GTG TTG CCT GC	ACV96791	Ping et al. (2011)
Epinecidin-1	Fw: CAT CGC CCT CTT TCT TGT GTT G Rev: CCC TCC CCG GGT TCA G	BQ096584	Pan et al. (2007)
IgM	Fw: ACC GTG ACC CTG ACT TGC TAT G Rev: CCC GAT GGA CCT GAC AAT AGC	AY875500	Cui et al. (2010)

or probiotic diets were analyzed by PCR-DGGE (Fig. 1). The DGGE profiles revealed complex and highly reproducible bacterial communities in the 3 individual fish of each treatment. Different DGGE patterns were observed in samples collected from the probiotic treatments and the control. Six DGGE bands (bands 1, 5, 7, 8, 9 and 18) were common to all samples from the control and both probiotic treatments, 6 bands (bands 2, 4, 11, 12, 15 and 17) were present only in the control group, while bands 3 and 10 were present only in treatment T1 (viable probiotic), and band 16 only in treatment T2 (heat-inactivated probiotic) (Fig. 1), suggesting a decrease in bacterial diversity in the 2 probiotic treatments. A dendrogram representing the similarity of the microbial profiles from the PCR-DGGE fingerprints is displayed in Fig. 2, which was in accordance with the DGGE profiles and demonstrated that the 2 probiotic triplicates were generally clustered into 1 group distinctly different from the control triplicate with the exception of 1 sample (fish D3) in treatment T2.

Eighteen predominant bands (bands 1 to 18) that appeared on the DGGE gel were excised and 13 bands were successfully sequenced. The resulting sequences were compared with data from the NCBI using the BLAST sequence algorithm and the results are shown in Table 2. Bands 2, 4, 11, 12 and 17 were present only in the control group, and

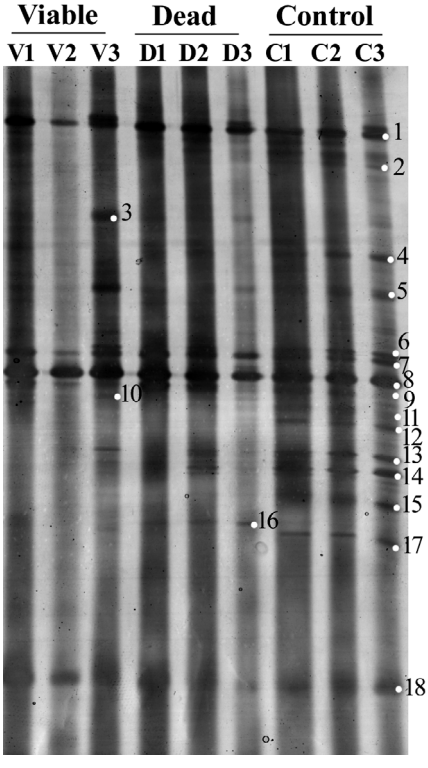

Fig. 1. PCR-DGGE fingerprints showing diversity of autochthonous intestinal microbiota of grouper *Epinephelus coioides* fed the control diet (C1, C2 and C3), viable *Bacillus pumilus* SE5 (V1, V2 and V3) and heat-inactivated SE5 (D1, D2 and D3)

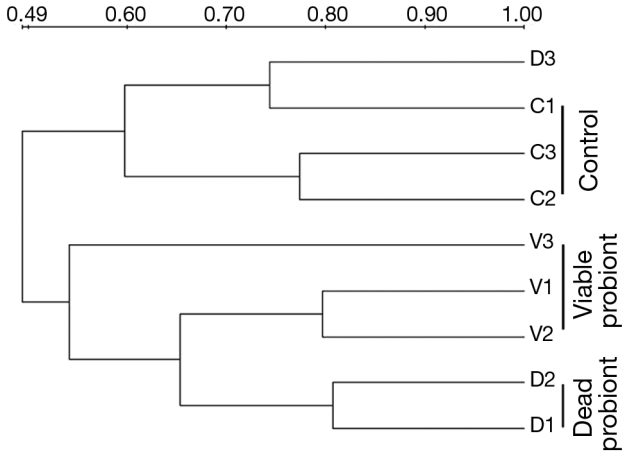

Fig. 2. Similarity dendrogram of PCR-DGGE fingerprints of autochthonous intestinal microbiota of grouper *Epinephelus coioides* fed the control diet (C1, C2 and C3), viable *Bacillus pumilus* SE5 (V1, V2 and V3) and heat-inactivated SE5 (D1, D2 and D3)

most closely related to uncultured *Shigella* sp. isolate DGGE gel band GYC43-3-like, *Psychroserpens burtonensis* ACAM181-like, uncultured gamma proteobacterium clone Fuku2-SW-PH56-like, *Pantoea agglomerans* isolate EB17-like and uncultured bacterium clone 080521-40-like bacterium, respectively. Bands 3 and 10 were only present in the T1 treatment and were closely related to *Photobacterium* sp. HDC28-like and uncultured bacterium isolate DGGE gel band 5-5-like bacterium, respectively, while band 16 was only present in the T2 treatment and showed 99% similarity to uncultured gamma proteobacterium clone 06ICW-like bacterium.

Expression of mucosal immune genes

TLR expression data acquired from RT-qPCR are presented in Fig. 3, which demonstrates that the TLR1 expression in the T1 treatment was upregulated significantly compared with the control ($p < 0.05$), while no significant difference was observed in the T2 treatment. The TLR2 expression in treatments T1 and T2 increased significantly compared with the control ($p < 0.05$), while the TLR5 expression in treatments T1 and T2 showed a significant downregulation ($p < 0.05$).

Adaptor MyD88 involved in the TLR signalling pathways has been shown to play an important role in resistance to bacterial infections. In this study, the expression of MyD88 in treatments T1 and T2 showed no significant difference compared with the control (Fig. 4).

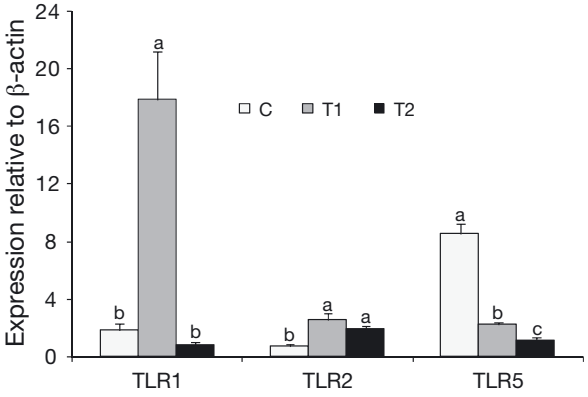

Fig. 3. Expression of toll-like receptors (TLR1, TLR2 and TLR5) in the intestine of grouper *Epinephelus coioides* fed the control diet (C), viable probiotic *Bacillus pumilus* SE5 (T1) and heat-inactivated SE5 (T2) for 60 d. Each bar represents the mean (±SE) value from 6 determinations (n = 6). Data with different letters are significantly different ($p < 0.05$)

Table 2. Closest relatives of intestinal bacteria in grouper *Epinephelus coioides* fed control, viable and heat-inactivated *Bacillus pumilus* SE5 as determined by a BLAST search of sequences generated for the bands in the DGGE gel (see Fig. 1). Percentage similarities to the closest relatives, and their accession numbers, are given

Band no.	Accession no.	Closest relative	Similarity (%)
1	KC991221	Uncultured *Escherichia* sp. isolate DGGE band N5	100
2	KC991203	Uncultured *Shigella* sp. isolate DGGE gel band GYC43-3	100
3	KC991217	*Photobacterium* sp. HDC28	99
4	KC991223	*Psychroserpens burtonensis* ACAM181	99
7	KC991208	*Enterobacter* sp. CTSP22	92
9	KC991215	Marine bacterium HC-17	100
10	KC991222	Uncultured bacterium isolate DGGE gel band 5-5	89
11	KC991233	Uncultured gamma proteobacterium clone Fuku2-SW-PH56	97
12	KC991234	*Pantoea agglomerans* isolate EB17	93
13	KC991225	Uncultured bacterium clone F1Q32TO03DV1RL	100
16	KC991229	Uncultured gamma proteobacterium clone 06ICW	99
17	KC991211	Uncultured bacterium clone 080521-40	100
18	KC991212	*Vibrio ruber* strain GHt9-5	100

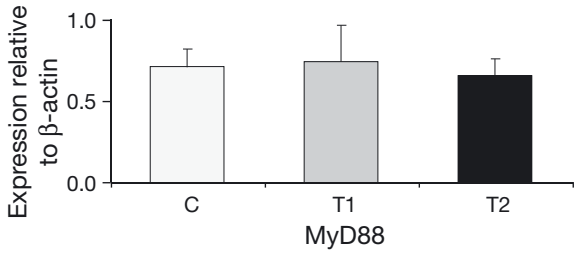

Fig. 4. The expression of adaptor MyD88 in the intestine of grouper *Epinephelus coioides* fed the control diet (C), viable probiotic *Bacillus pumilus* SE5 (T1) and heat-inactivated SE5 (T2) for 60 d

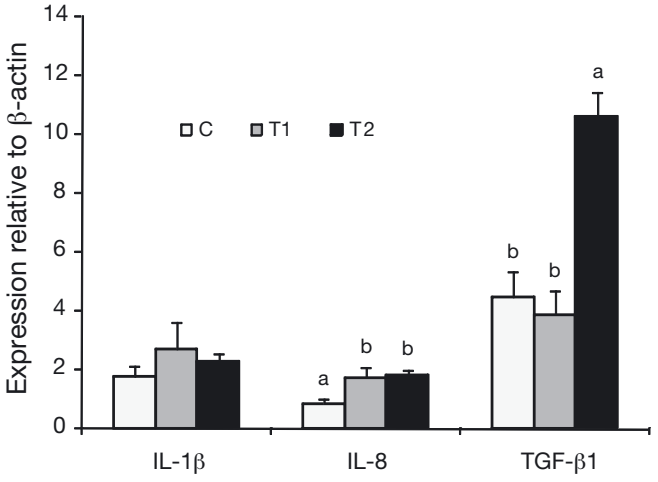

Fig. 5. The expression of cytokines (IL-1β and IL-8 and TGF-β1) in the intestine of grouper *Epinephelus coioides* fed the control diet (C), viable probiotic *Bacillus pumilus* SE5 (T1) and heat-inactivated SE5 (T2) for 60 d. Each bar represents the mean (±SE) value from 6 determinations (n = 6). Data with different letters are significantly different ($p < 0.05$)

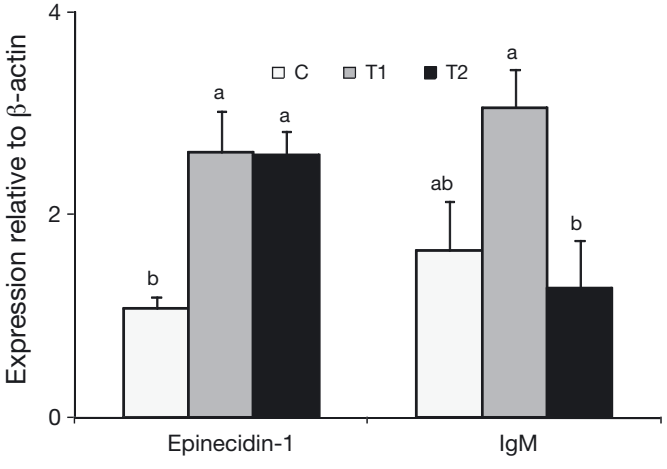

Fig. 6. The expression of antibacterial proteins (epinecidin-1 and IgM) in the intestine of grouper *Epinephelus coioides* fed the control diet (C), viable probiotic *Bacillus pumilus* SE5 (T1) and heat-inactivated SE5 (T2) for 60 d. Each bar represents the mean (±SE) value from 6 determinations (n = 6). Data with different letters are significantly different ($p < 0.05$)

The expression of cytokine genes, including the pro-inflammatory cytokines (IL-1β and IL-8) and anti-inflammatory cytokine (TGF-β1), was assessed by RT-qPCR (Fig. 5). In treatment T1, IL-8 expression increased significantly ($p < 0.05$), while IL-1β and TGF-β1 expression showed no significant difference compared with the control. In treatment T2, IL-8 and TGF-β1 expression showed a significant upregulation ($p < 0.05$), while IL-1β expression increased slightly, but was not statistically significant.

The expression of genes for 2 antibacterial proteins, epinecidin-1 and IgM, was determined by RT-qPCR (Fig. 6). The expression of epinecidin-1 in treatments T1 and T2 was enhanced significantly compared with control ($p < 0.05$), while increased IgM expression was observed in treatment T1, but the increase was not statistically significant.

DISCUSSION

It has been extensively demonstrated that viable probiotics can shape the gut microbiota in fish (Tapia-Paniagua et al. 2010, Ferguson et al. 2010, Sun et al. 2011, Liu et al. 2013), but little attention has been paid to the impact of dead probiotics on the microbial community (Hoseinifar et al. 2011, Mohapatra et al. 2012). Using cultivation-based techniques, Hoseinifar et al. (2011) reported that dietary administration of commercial inactive brewer's yeast *Saccharomyces cerevisiae* var. *ellipsoideus* elevated the level of lactic acid bacteria, the most common types of beneficial microorganisms in the gut of fish (Ringø et al. 2010), and that this could positively affect host health and immunity. Interestingly, both the viable and heat-killed mixed probiotics (*Bacillus subtilis*, *Lactococcus lactis* and *S. cerevisiae*) significantly reduced the total heterotrophic bacterial population in the intestine of *Labeo rohita*, but the response was more rapid and substantial with the viable probiotics (Mohapatra et al. 2012). In line with the previous studies, both the viable and heat-inactivated *Bacillus pumilus* SE5 decreased the diversity of dominant intestinal microbial populations, as several bacteria (corresponding to bands 2, 4, 11, 12, 15 and 17) were suppressed to undetectable levels in the intestine of *Epinephelus coioides*. Among those suppressed bacteria, *Psychroserpens burtonensis* (corresponding to band 4) has been suggested as a potential opportunistic pathogen associated with salmonid amoebic gill disease (Bowman & Nowak 2004), while *Pantoea agglomerans* (corresponding to band 12) is a known enteric pathogenic bacterium to fish (Hansen et al.

1990, Austin & Austin 2012). These results suggest that both the viable and heat-inactivated *B. pumilus* SE5 controlled those potentially pathogenic bacteria, and this may benefit the health of host. Obviously, the heat-inactivated probiotic cannot suppress pathogenic bacteria by competition for nutritional substances or secretion of inhibitory substances. Therefore, we presume that the most likely mechanism whereby the heat-inactivated probiotic modulates the gut microbiota is by means of activating the mucosal immunity of fish, as several heat-inactivated probiotics have exhibited promising mucosal immunomodulatory activity in various fish species (Nayak 2010, Pérez et al. 2010).

It is generally accepted that TLR signalling pathways in mammals play essential roles in the recognition of the probiotics and activation of the mucosal immune system (Abreu 2010, Sánchez de Medina et al. 2013). However, the roles of fish TLRs in probiotic-induced mucosal immune response have received relatively little attention until recently (Pérez et al. 2010). In this study, TLR1, TLR2 and TLR5, which have been suggested to be involved in probiotic recognition and mucosal immune activation in homothermic animals (Sánchez de Medina et al. 2013), were determined by RT-qPCR. Significantly elevated expression of TLR1 and TLR2 was observed in fish fed the viable *B. pumilus* SE5, while the expression of TLR2 was upregulated in fish fed the heat-inactivated SE5. The enhanced TLR2 expression induced by both the viable and heat-inactivated SE5 may result from the interaction of the host with the probiotic MAMPs, such as lipoprotein/lipopeptides, peptidoglycan and lipoteichoic acid (Sánchez de Medina et al. 2013). Interestingly, we noticed that the heat-inactivated SE5 failed to upregulate the expression of TLR1, suggesting that heat treatment may affect the probiotic MAMPs and impair the efficacy of the probiotic. Moreover, the expression of TLR5 decreased significantly in fish fed both the viable and heat-inactivated *B. pumilus* SE5. While the exact mechanisms are not clear, we proposed 2 possible reasons. Firstly, TLR5 reacts only with flagellin, (Sánchez de Medina et al. 2013). *B. pumilus* SE5 does not have flagellin and therefore has no impact on the expression of TLR5. Secondly, both the viable and heat-inactivated *B. pumilus* SE5 have been shown to decrease the levels of several bacteria with flagella, such as *Shigella* sp. (corresponding to band 2) and *P. agglomerans* (corresponding to band 12) (Holt 1994, Girón 1995), which may tune the expression of TLR5.

Although the expression of adaptor MyD88 was not affected, the expression of pro-inflammatory cytokine (IL-8) was upregulated in fish fed the viable *B. pumilus* SE5, while the expression of IL-8 and anti-inflammatory immune gene (TGF-β1) was enhanced in fish fed the heat-inactivated SE5. In line with our results, the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N) and anti-inflammatory cytokines (IL-10 and TGF-β1) in Japanese pufferfish *Takifugu rubripes* head kidney (HK) cells was generally upregulated after 1, 4, 8, 12, 24 and 48 h of incubation with 2 heat-killed bacteria, namely *Lactobacillus paracasei* spp. *paracasei* (strain 06TCa22) and *L. plantarum* (strain 06CC2) (Biswas et al. 2013). In contrast, Lazado et al. (2010) reported that after 3 h of incubation with Atlantic cod *Gadus morhua* HK leukocytes, viable *Psychrobacter* sp. GP12 upregulated the expression of IL-1β and IL-8, but the heat-inactivated GP12 failed to do so. Therefore, different probiotic strains and different forms of one probiotic may exert different impacts on the expression of cytokines in fish, and future studies should pay more attention to the molecular interactions between the probiotics and host immune system.

Teleost fish possess a rich repertoire of antimicrobial peptides (AMPs), which are able to kill pathogens by interacting directly with their negatively charged membranes, disrupting the osmotic balance of the microbial membrane (Pan et al. 2007, Broekman et al. 2013). *In vitro* studies showed that the dead autochthonous probiotic *Lactobacillus* sp. elicited a significant expression of cathelicidin in the Atlantic cod cell line (Broekman et al. 2013). In line with the previous study, our *in vivo* study demonstrated that the expression of epinecidin-1 was upregulated in the intestine of *E. coioides* fed both the viable and heat-inactivated *B. pumilus* SE5, and this is consistent with intestinal microbiota data showing that several bacteria (including potentially pathogenic *P. burtonensis* and *P. agglomerans*) decreased to undetectable levels in fish fed both the viable and heat-inactivated probiotic. As the heat-inactivated probiotic could not modulate the intestinal microbiota by mechanisms such as competition for nutritional substances and secretion of inhibitory substances, we speculate that the probiotic-induced activation of intestinal mucosal immunity, especially the significantly upregulated expression of antibacterial peptides, may play an important role in the intestinal microbiota modulation.

In conclusion, both the viable and heat-inactivated probiotic *B. pumilus* SE5 shape the intestinal microbiota and mucosal immune gene expression in grouper *E. coioides*. The probiotic-induced activation

of intestinal mucosal immunity, especially the activation of antibacterial epinecidin-1, may play an important role in the intestinal microbiota modulation in *E. coioides*. This work lays the foundation for future studies on the molecular interactions between probiotics, gut microbiota and mucosal immunity in fish.

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