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Bacillus pumilus SE5 originated PG and LTA tuned the intestinal TLRs/MyD88 signaling and microbiota in grouper (*Epinephelus coioides*)

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ABSTRACT

The normal microbiota plays a key role in the health of host, but little is known of how the fish immune system recognizes and responds to indigenous bacteria/probiotics. Our previous studies have showed that heat-inactivated indigenous *Bacillus pumilus* SE5 activate the TLR2 signaling pathways and modulate the intestinal microbiota in grouper (*Epinephelus coioides*), suggesting microbial-associated molecular patterns (MAMPs) involved. In this study, whole cell wall (CW) and two possible MAMPs, peptidoglycan (PG) and lipoteichoic acid (LTA) have been extracted from *B. pumilus* SE5 and their effects on intestinal immune related genes expression and microbiota were evaluated in a 60 days feeding trial. Significantly elevated expression of TLR1, TLR2, TLR5 and MyD88 was observed in fish fed the CW, PG and LTA containing diets, and the highest expression was observed in groups PG and LTA. At the same time, significantly upregulated expression of antimicrobial effectors, such as antimicrobial peptides (epinecidin-1, hepcidin-1 and β-defensin), C-type Lectin and IgM was observed in fish fed PG and LTA containing diets. This induced activation of intestinal immunity was consistent with the microbiota data showing that CW, PG and LTA originated from SE5 modulated the overall structure of intestinal microbiota, and the relative abundance of potentially pathogenic *Vibrio* decreased significantly while beneficial *Lactobacillus* increased significantly in fish fed PG and LTA. In conclusion, both the PG and LTA originated from *B. pumilus* SE5 could activate TLRs/MyD88 signaling and expression of wide-ranging antibacterial effectors, and therefore shape the intestinal microbiota in grouper.

1. Introduction

Aquaculture is a fast growing and very important high-quality animal protein producing industry in the world, but one problem we had to face is that large amounts of antibiotics are often used for disease prevention and control, owing to the high mortality and contagious nature of bacterial diseases in aquaculture. It has been widely recognized that the use of antibiotics as a preventive measure may alter the gut microbiota of fish and induce resistant bacterial populations, with unpredictable long-term effects on public health [1]. Therefore, there is a growing and strong interest on the use of probiotics as a sustainable alternative for improving the general health and welfare in fish [1,2]. Recently, more and more scientists and farmers realized that intestinal indigenous beneficial bacteria have many advantages to be explored as probiotics in fish aquaculture [2,3]. Pitifully, little information is available about the molecular interactions between indigenous bacteria/probiotics, intestinal microbiota and mucosal immunity in fish, this certainly limits the selection and application of intestinal indigenous probiotics [2,3].

Fish intestinal mucosal surfaces interface with a complex and dynamic community of microorganisms, which is reported to play important roles within the intestinal tract, including the capacity to contribute to the development/maturation of the intestinal mucosal immune system [3]. On the other hand, the intestinal mucosal immune system has been suggested to surveille and shape the microbial populations [2,4]. Probiotics, usually members of the normal indigenous microbiota, which play an important role in the health of animals, and their application can assist in returning a disturbed microbiota to its normal beneficial composition [1]. Although lack of data, it has been proposed that pattern-recognition receptors (PPRs) such as toll-like receptors (TLRs) and nucleotide-binding and oligomerisation domains (NOD)-like receptors (NLRs) may recognize microbial-associated

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molecular patterns (MAMPs) shed by commensal microbes/probiotics and activate signaling cascades that finely tune the production of cytokines and antimicrobial proteins, this will benefit the establishment of a balanced intestinal microbial community in fish [2,4,5]. Our previous studies have showed that heat-inactivated indigenous probiotic *Bacillus pumilus* SE5 activate the TLR2 signaling pathways and modulate the intestinal microbiota in grouper (*Epinephelus coioides*), and suggesting the interaction of host with the probiotic MAMPs [6]. To illustrate this postulation, in this study, cell wall (CW), and two possible MAMPs, peptidoglycan (PG) and lipoteichoic acid (LTA) have been extracted from *B. pumilus* SE5 and their effects on expression of TLRs signaling related genes and intestinal microbiota were evaluated.

2. Materials and methods

2.1. Probiotic strain and cell wall components extraction

Probiotic *B. pumilus* SE5 was isolated from the intestine of juvenile grouper *E. coioides* [7], and cultured and prepared as described previously [6,7]. After incubation, the cells were harvested and re-suspended in PBS. The number of bacteria in the suspension was determined by plate counting on tryptone soya agar (TSA) at 28 °C for 48 h, and then the live bacterial suspension was heat-inactivated in a water bath at temperature 95 °C for 60 min, and the non-viability was checked by plating on TSA.

Extraction of cell wall from *B. pumilus* SE5 was carried out as described in Ref. [8] with modification. Bacterial culture was submitted to centrifugation at 10,000 × *g* for 15 min and then the cells were washed with sterile PBS. The harvested cells were suspended in a lysis solution (0.02 mol/L PBS, 1 mmol/L Phenylmethanesulfonyl fluoride, pH 7.4) and submitted to sonication for 20 min, with 2 min intervals on ice with a sonicator JY 92-IIN (Ningbo New Art Ultrasonic Equipment Co., Ltd, China). The target product was harvested by centrifugation at 8000 × *g* for 10 min and washed with sterile water for 3–4 times, then lyophilized and stored at −80 °C.

The extraction of PG from *B. pumilus* SE5 was based on a TCA method as described by Ref. [9] with slightly modification. Briefly, bacterial sludge was dissolved in 10% TCA (w/v = 1:10), incubated in a boiling bath for 1 h, and then centrifuged at 13,000 × *g* for 10 min. The sediment was collected and treated with a special solvent (Acetic acid-sodium acetate buffer (0.5 M acetic acid and 0.2 M sodium acetate, pH 4.5), chloroform, methanol mix with the ratio of 4:5:10 (v/v/v), pH 4.6) for 24 h. After centrifugation at 8000 × *g* for 20 min, the insoluble residue was incubated in Tris-HCL (0.1 M, pH 7.5) containing 0.15% trypsin at 37 °C in a shaking bath (140 rpm) for 12 h. Finally, the mixture was centrifuged at 8000 × *g* for 20 min and the sediment was harvested and washed in sterile water for 5–6 times, then lyophilized and stored at −80 °C for target product analysis as described in Ref. [9].

Extraction of LTA from *B. pumilus* SE5 was carried out according to Ref. [10] with slightly modification. Briefly, bacteria suspension was mixed with an equal volume of n-butanol (Xilong Chemical, Shantou, China) in a shaking bath (170 rpm) for 1 h at room temperature. After centrifugation at 6800*g* for 20 min, the aquatic phase was lyophilized, resuspended with chromatography start buffer (15% n-propanol in 0.1 M ammonium acetate, pH 4.7), and centrifuged at 6800 g for 20 min. The supernatant was subjected to hydrophobic interaction chromatography (HIC) on octyl-Sepharose. The target product was analyzed and confirmed as described in Ref. [10].

2.2. Diet preparation and experimental design

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 [6,11], and shaping the gut microbiota of grouper *E. coioides* [6]. In the present study, therefore, cell wall components were extracted from the same dose (1.0×10^8 CFU g^{-1}) of SE5 and their effects on intestinal microbiota and immune genes expression in *E. coioides* were evaluated. The basal diet was formulated as described in Ref. [6]. The experiment diets were prepared by gently spraying the required amount of heat-inactivated SE5, CW, PG or LTA suspensions on the control diet and mixed in a three dimensional drum mixer (SYH-100, Punaier Drying Equipment Co., Ltd, Changzhou, China). Dietary ingredients of each diet 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.

The animal trial was conducted in the Haikang Aquaculture Research Base of Dabeilong Aquaculture group followed the protocols approved by animal care and use committee of Jimei University, China. The feeding experiment was conducted in 20 tanks containing 300 l seawater, each tank connected to an open circulating system (30 g l^{-1} salinity, at 26 ± 2 °C). Each tank was randomly stocked with 30 fish (14.57 ± 0.05 g) obtained from the Haikang Aquaculture Research Base of Dabeilong Aquaculture group and each treatment was conducted in quadruplicate. The fish were fed to apparent satiation at 08:30 and 18:30 h with the control diet, heat-inactivated SE5, CW, PG and LTA containing diets, respectively. At the end of the trial (day 60), nine fish were taken randomly from each tank, and the intestine of each fish was aseptically excised and the digesta was removed under sterile conditions as described by Ref. [6]. The intestines of three fish in each tank were randomly put together as one sample, four intestinal samples per treatment were kept in Eppendorf tubes at −80 °C for DNA extraction and microbial analysis, and the other eight intestinal samples per treatment were stored at −80 °C in TRIzol reagent (Invitrogen, Carlsbad, USA) for RNA extraction and immune genes analysis.

2.3. Intestinal microbiota analysis

Total DNA was extracted from the intestinal samples as described previously [12]. The quantity and purity of the DNA were assessed using a Nano-Drop®ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The integrity of the extracted genomic DNA was determined by electrophoresis on a 1.2% (w/v) agarose gel. The V3-V4 region of the 16S rRNA gene of intestinal bacteria, which has been confirmed to provide ample information for taxonomic classification of microbial communities [3], was amplified with the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Thermal cycling consisted of the following condition: 95 °C for 5 min (1 cycle), 95 °C for 30 s/50 °C for 60 s/72 °C for 60 s (15 cycles), and a final extension at 72 °C for 7 min. The quantity and purity of the PCR product were assessed and high-throughput sequencing was performed on an Illumina MiSeq platform at Beijing Biomarker Biotechnology Co. Ltd (Beijing, China).

The raw paired-end reads from the original DNA fragments were merged using FLASH [13], and assigned to each sample according to the unique barcodes. High-quality reads for bioinformatics analysis were performed and all of the effective reads from each sample were clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity according to UCLUST [14]. For beta diversity analysis, principal component analysis (PCA), and unweighted pair group method with arithmetic mean (UPGMA) were performed using QIIME (v1.7.0) and displayed with R software (v2.15.3) [15]. To assess the changes in microbial community structure brought by different diets, Metastats analysis was used to identify significant differences in dominant genera between the control and treatments as described in Ref. [16].

2.4. Immune genes expression analysis

Total RNA was extracted from intestinal tissues as our previously described [6,17]. The immune related genes, such as TLR1, TLR2,

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

Gene	Nucleotide sequence (5′-3′)	Accession No.	Reference
β-actin	Fw = GACATCAAGGAGAAGCTGTG Rev = TGCTGTTGTAGGTGGTCTCGT	AY510710	[6]
TLR1	Fw = CCAGGGTCGCAGAGTCCTATC Rev = GCCAGCCAAGTTCAGTTTCGT	HM357229	[6]
TLR2	Fw = AGGGTTCAGAAGGGTTGCTAT Rev = CAGGAAGGAAGTCCCGTTTGT	HM357230	[6]
TLR5	Fw = CTGACCCTGATGCTTTTCG Rev = GCTACTTTACTGCTGTGTG	GH612592	[6]
MyD88	Fw = AGCTGGAGCAGACGGAGTG Rev = GAGGCTGAGAGCAAACTTGGTC	GQ202584	[6]
Epinecidin-1	Fw = CATCGCCCTCTTTCTTGTGTTG Rev = CCCTCCCCGGGTTCAG	BQ096584	[6]
Hepcidin-1	Fw = CTGACAAGAGGCACCAAG Rev = TGAAGGCGAGCACGAT	GU391243.1	
β-defensin	Fw = ATGAAGGGACTGAGCTTGGTTC Rev = CATCTGTATCTCCGAGGGCAACCGT	JN709461	[18]
C-type Lectin	Fw = ATCGCATAACAGAGCCAGAC Rev = CAGGGAACATCACTCCAAAC	FJ805452	[19]
IgM	Fw = ACCGTGACCCTGACTTGCTATG Rev = CCCGATGGACCTGACAATAGC	AY875500	[6]

TLR5, MyD88, epinecidin-1 and IgM were determined as our previously described [6,17]. In addition, another three immune effectors, β-defensin and C-type Lectin were determined using RT-qPCR with specific primers as previously reported (Table 1), while hepcidin-1 with primers designed using Primer 5.0 based on the whole nucleotide sequence of the gene (accession number: GU391243.1). The RT-qPCR was performed with the SYBR Green Realtime PCR Master Mix (Toyobo, Shanghai, China) in an ABI 7500 real-time PCR Detection system (Applied Biosystems, California, USA). 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 three times.

2.5. Data statistical analysis

Data of expression of immune genes from eight samples are presented as fold increase (mean ± standard error, SE). Data were examined by one-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 19.0 (SPSS Inc., Chicago, IL, USA). Significant differences were declared at $P \leq 0.05$.

3. Results

3.1. PG and LTA shape the intestinal microbiota

At phylum level, Proteobacteria, Firmicutes, Bacteroidetes, Cyanobacteria and Actinobacteria were detected as the most predominant bacterial phyla in the intestine of grouper from all groups, and decreased Proteobacteria and increased Cyanobacteria, Firmicutes, Bacteroidetes and Actinobacteria relative abundances were observed in all treatments compared with the control (Fig. 1A). At genus level, *Vibrio*, *Photobacterium*, *Serratia*, *Bacteroides*, *Lactobacillus*, *Lachnospiraceae*, *Pseudomonas*, *Lachnoclostridium* and *Prevotella* composed the most dominant genera in the intestine of grouper, and decreased *Vibrio* and *Lachnospiraceae*, while increased *Lactobacillus* and *Bacteroides* relative abundances were observed in all treatments compared with the control. In addition, greatly decreased *Photobacterium* relative abundance was observed in groups CW and PG (Fig. 1B).

The Principal component analysis (PCA) and phylogenetic tree based on binary_jaccard distance were used to compare the microbial similarities of fish fed different diets. The results showed that all the treatments samples showed a clear separation with the control at genus level (Fig. 2), indicating that *B. pumilus* SE5 originated CW, PG and LTA modulated the overall structure of intestinal microbiota in grouper. MetaStat analysis showed significant differences in several dominant genera between the treatments and control. Significantly improved relative abundance of *Lactobacillus* was observed in all treatments compared with the control ($P < 0.05$) (Table 2). The inclusion of PG and LTA in diets significantly decreased the relative abundance of *Vibrio* ($P < 0.05$), CW and PG significantly decreased the relative abundance of *Photobacterium* ($P < 0.05$), while HK, CW and PG significantly enhanced the relative abundance of *Methylobacterium* ($P < 0.05$) (Table 2).

3.2. PG and LTA modulate the immune-related genes expression

TLR1, TLR2, TLR5 and MyD88 expression data acquired from RT-qPCR are presented in Fig. 3, significantly upregulated expression of TLR1 was observed in all treatments compared with the control, and the expression of TLR1 in groups PG and LTA was higher significantly than that in groups HK and CW ($P < 0.05$). Significantly upregulated expression of TLR2 and TLR5 was observed in groups CW, PG and LTA compared with the control and group HK (Fig. 3A) ($P < 0.05$), and the highest expression of TLR2 and TLR5 was observed in groups PG and LTA. The expression of adaptor MyD88 in all the treatments improved significantly compared with the control, and the expression of MyD88 in groups CW, PG and LTA was higher significantly than that in group HK ($P < 0.05$) (Fig. 3B).

The expression of genes for immune effectors, including antibacterial peptides (epinecidin-1, hepcidin-1 and β-defensin), C-type Lectin and IgM, was determined by RT-qPCR (Fig. 4). Compared with control, significantly increased expression of epinecidin-1, hepcidin-1 and IgM was observed in all the treatment, and significantly increased expression of β-defensin and C-type Lectin in groups CW, PG and LTA ($P < 0.05$), and the highest expression of all immune effectors was observed in groups PG and LTA (Fig. 4).

4. Discussion

The intestinal microbiota of fish contributes to digestion and affects the growth, reproduction and vulnerability of the host to diseases [3].

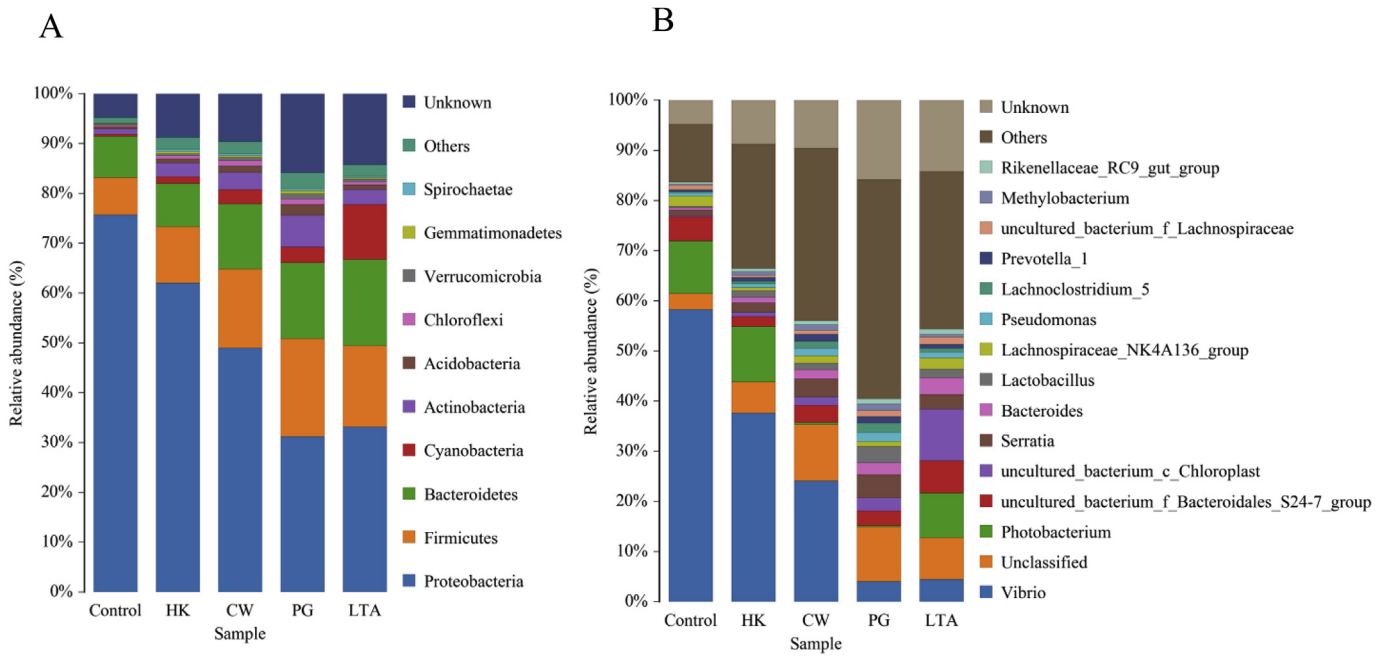

Fig. 1. Taxonomy classification of reads from 16 S rRNA V3–V4 regions of intestinal bacteria at phylum (A) and genus (B) taxonomic levels. Only top 15 most abundant phyla and genera (Based on relative abundance) were shown in the figures. Other phyla and genera were all assigned as 'Others'.

The indigenous microbes/probiotics have been suggested to activate the intestinal immunity [6,17,20] and shape the intestinal microbiota in fish [6,21–23]. The complex relationship between host and the intestinal normal microbiota presents an intriguing question how the intestinal immune system recognizes and reacts with the indigenous microbes/probiotics and modulate the intestinal microbiota. In this study, cell wall (CW), peptidoglycan (PG) and lipoteichoic acid (LTA) have been extracted from the indigenous probiotic *B. pumilus* SE5 and their effects on intestinal microbiota and TLRs signaling genes expression in grouper (*E. coioides*) were evaluated. The results indicated that CW, PG and LTA originated from probiotic *B. pumilus* SE5 modulated the overall structure of intestinal microbiota in grouper (Figs. 1 and 2), and significantly improved the relative abundance of common beneficial *Lactobacillus* in fish (Fig. 1, Table 2) [24,25]. On the other hand, dietary PG and LTA significantly decreased the relative abundance of common pathogenic *Vibrio* in marine fish [26], while CW and PG containing diets significantly decreased the relative abundance of *Photobacterium* (Fig. 1, Table 2). Although genus *Photobacterium* are not common pathogens in fish, some non-luminous species such as *Photobacterium damselae*, *Photobacterium rosenbergii*, *Photobacterium jeanii* and *Photobacterium iliopiscarium* were found to be associated with numerous diseases in fish [27]. Therefore, CW and especially PG and LTA originated from *B. pumilus* SE5 could positively shape the intestinal microbiota and this will certainly benefit the health of the host fish. In the following paragraphs we will discussed how the intestinal immune system recognizes and reacts with the indigenous *B. pumilus* SE5 and

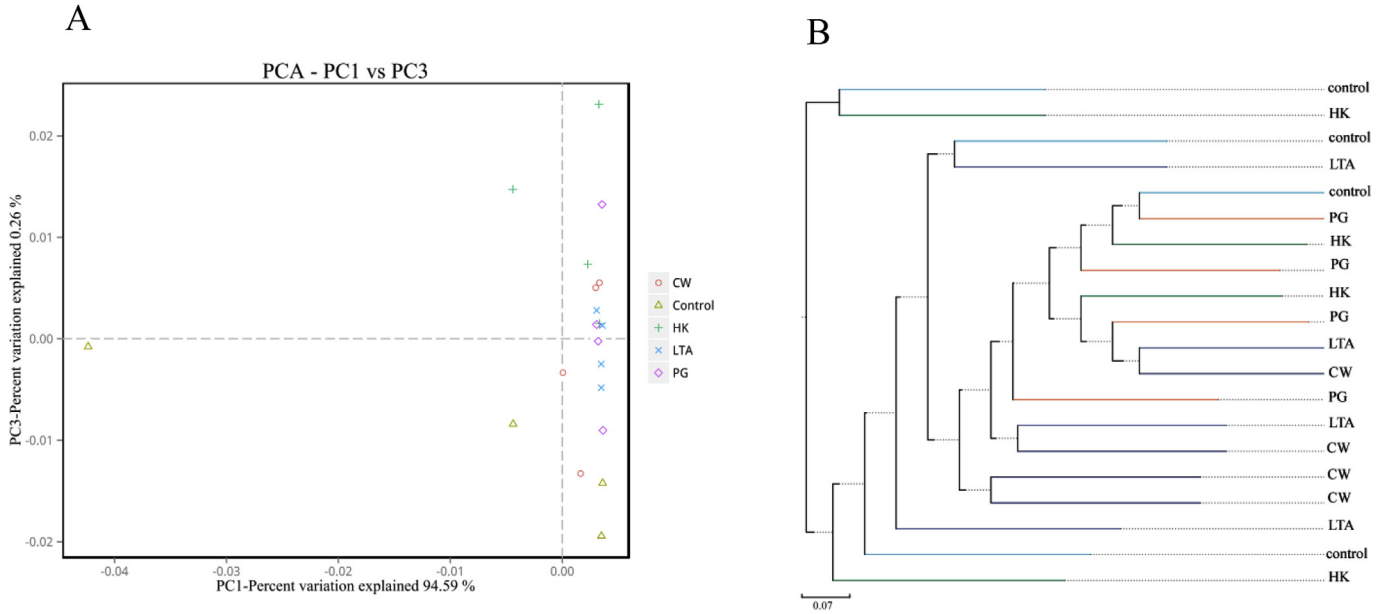

Fig. 2. Beta diversity of intestinal microbiota of grouper *E. coioides*. (A) Principal Component Analysis (PCA) against PC1 versus PC3 axes based on genus; (B) Samples UPGMA-clustering trees based on binary_jaccard distance at genus level.

Table 2
Metastats analysis of the abundance of bacterial genera ($\times 10^{-4}$) in the intestine of grouper *E. coioides*. Only the most dominant bacterial genera (Based on relative abundance as shown in Fig. 1B) were shown in the table.

Genus	Control	HK	CW	PG	LTA
Vibrio	3090 ± 1230a	2870 ± 810a	1780 ± 619a	357 ± 78b	414 ± 68b
Photobacterium	794 ± 757a	1180 ± 1140a	36 ± 9.79b	26.5 ± 18.2b	853 ± 798a
Serratia	238 ± 128	198 ± 79.9	310 ± 52.3	394 ± 50.5	255 ± 24.9
Bacteroides	84.2 ± 31.9	102 ± 5.22	172 ± 39.2	204 ± 36.9	311 ± 137
Lactobacillus	38.4 ± 16.1b	134 ± 40.7a	104 ± 21.6a	272 ± 100a	151 ± 22.6a
Pseudomonas	139 ± 74.6	70.6 ± 24.1	123 ± 20.4	156 ± 26.7	104 ± 15.1
Lachnoclostridium	76.9 ± 42.4	53.2 ± 11	117 ± 25.4	154 ± 30.3	66.3 ± 2.72
Prevotella	64.3 ± 32.4	77.1 ± 20.5	122 ± 6.21	117 ± 15.7	69.9 ± 11.4
Methylobacterium	42.3 ± 21.2b	83.1 ± 23.8a	104 ± 7.58a	114 ± 16.8a	45.6 ± 9.34b

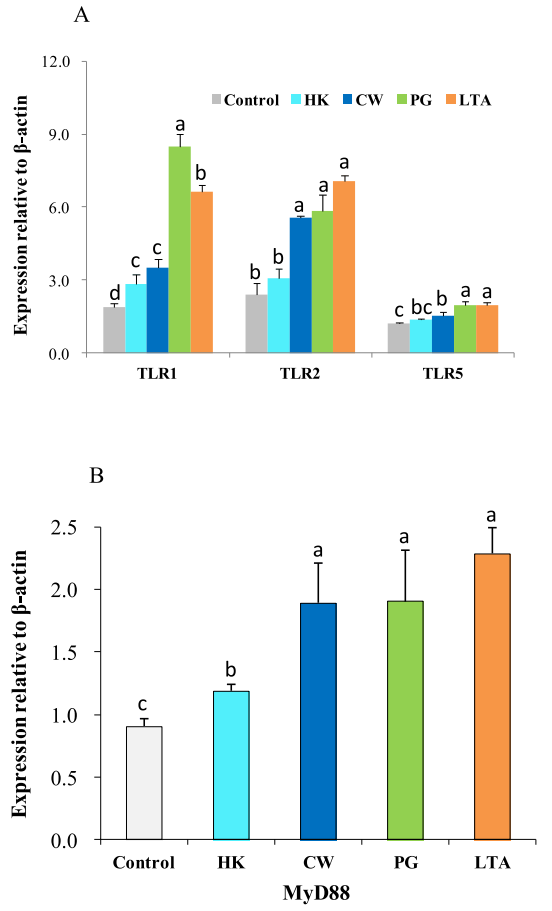

Fig. 3. The expression of Toll-like receptors (TLR1, TLR2 and TLR5) (A) and adaptor MyD88 (B) in the intestine of grouper *E. coioides*.

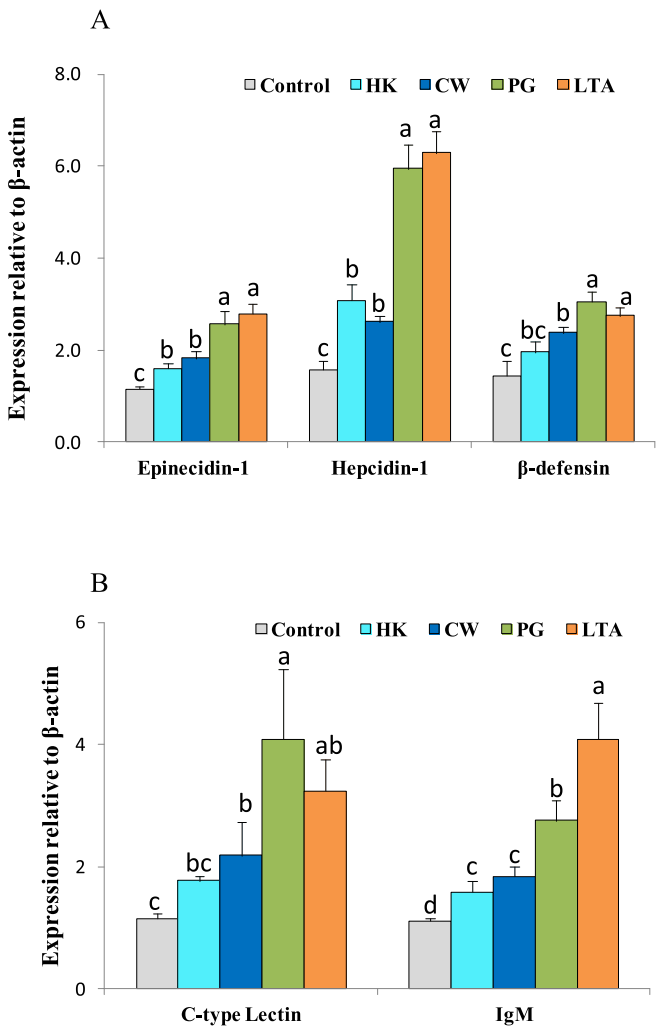

Fig. 4. The expression of (A) antibacterial peptides (epinecidin-1, hepcidin-1 and β-defensin), (B) C-type Lectin and IgM in the intestine of grouper *E. coioides*.

therefore modulate the intestinal microbiota.

TLRs signaling pathways may play essential roles in the recognition of indigenous microbes/probiotics and activation of the mucosal immune system in fish, however, this has received little attention until recently [6,17,20]. Recently, TLR2/MyD88 signaling has been reported to play a key role in innate immune recognition and activation during the colonization of two indigenous microbes (*Chryseobacterium* ZOR0023, belonging to phylum Bacteriodetes and *Exiguobacterium* ZWU0009, belonging to phylum Firmicutes) in zebrafish [20]. Similarly, our previous studies have showed that both the viable and heat-inactivated indigenous *B. pumilus* SE5 activate the TLR2 signaling pathways in grouper, and the enhanced TLR2 signaling may possibly result from the interaction of host with the probiotic MAMPs, such as lipoprotein/lipopeptides, PG and LTA [6,20]. To illustrate this postulation, in this study, two major possible MAMPs, PG and LTA have been extracted from the *B. pumilus* SE5 and their effects on TLRs signaling genes expression were evaluated. The expression of TLR1, TLR2 and TLR5, which have been suggested to be involved in gram-positive probiotic recognition and mucosal immune activation in homothermic animals [28], elevated significantly in fish fed the CW, PG and LTA containing diets, and the highest expression was observed in fish fed PG and LTA containing diets. Furthermore, the adaptor protein MyD88, which involved in the TLRs signaling pathways and play an important role in resistance to bacterial infections [20], improved significantly in

fish fed the CW, PG and LTA containing diets. This data illustrated that PG and LTA from the *B. pumilus* SE5 did act as MAMPs and activate the TLRs signaling pathways in grouper (*E. coioides*).

Teleost fish possess a rich repertoire of antimicrobial effectors, such as antimicrobial peptides (AMPs), C-type Lectin and immunoglobulins, which have been suggested to play a key role in controlling pathogens and maintaining homeostasis in the intestine of fish [5,29,30]. Several studies have showed that indigenous probiotics/microbes could induce the expression of antimicrobial effectors in fish [6,17,31]. For example, indigenous *Lactobacillus* sp. elicited a significant expression of an important AMP, cathelicidin in the Chinook salmon (*Oncorhynchus tshawytscha*) cell line [31]. Our previous in vivo studies demonstrated that heat-inactivated indigenous *B. pumilus* SE5 upregulated the expression of an AMP (epinecidin-1) [6], and heat-inactivated indigenous *Psychrobacter sp.* SE6 upregulated the expression of epinecidin-1 and IgM in the intestine of grouper *E. coioides* [17]. In this study, the CW, PG and LTA originated from *B. pumilus* SE5 upregulated the expression of three AMPs (epinecidin-1, hepcidin-1 and β-defensin) in grouper. In addition, upregulated expression of another two immune effectors (C-type Lectin and IgM) was observed in fish fed the PG and LTA, but not in fish fed CW containing diets. These results suggested CW and especially PG and LTA could effectively activate the expression of antimicrobial effectors, this was highly consistent with intestinal microbiota data showing that the relative abundance of potentially pathogenic *Vibrio* decreased significantly while beneficial *Lactobacillus* increased significantly in fish fed PG and LTA. Therefore, the PG and LTA originated from *B. pumilus* SE5 induced the expression of antibacterial proteins may play a key role in modulating the intestinal microbiota.

In conclusion, both the PG and LTA originated from probiotic *B. pumilus* SE5 could be recongnized by TLRs signaling, activate the expression of antibacterial effectors and therefore shape the intestinal microbiota in grouper *E. coioides*. However, which TLR play the key role in indigenous microbes/probiotics recognization? Which downstream signaling pathways will be activated, NF-κB or MAPK? This questions need to be clarified in future studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.005.

References

[1] D.L. Merrifield, A. Dimitroglou, A. Foey, S.J. Davies, R.T.M. Baker, J. Bøgwald, M. Castex, E. Ringø, The current status and future focus of probiotic and prebiotic applications for salmonids, Aquaculture 302 (2010) 1–18 https://doi.org/10.1016/j.aquaculture.2010.02.007.

[2] C.C. Lazado, C.M. Caipang, E.G. Estante, Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions, Fish Shellfish Immunol. 45 (1) (2015) 2–12 https://doi.org/10.1016/j.fsi.2015.02.023.

[3] A.R. Wang, C. Ran, E. Ringø, Z.G. Zhou, Progress in fish gastrointestinal microbiota research, Rev. Aquacult. 10 (2018) 626–640 https://doi.org/10.1111/raq.12191.

[4] T. Pérez, J.L. Balcázar, I. Ruiz-Zarzuela, N. Halaihel, D. Vendrell, I. de Blas, J.L. Múzquiz, Host-microbiota interactions within the fish intestinal ecosystem, Mucosal Immunol. 3 (2010) 355–360 https://doi.org/10.1038/mi.2010.12.

[5] J.H.W.M. Rombout, L. Abelli, S. Picchietti, G. Scapigliati, V. Kiron, Teleost intestinal immunology, Fish Shellfish Immunol. 31 (2011) 616–626 https://doi.org/10.1016/j.fsi.2010.09.001.

[6] H.L. Yang, H.Q. Xia, J.D. Ye, W.C. Zou, Y.Z. Sun, Probiotic *Bacillus pumilus* SE5 shapes the intestinal microbiota and mucosal immunity in grouper *Epinephelus coioides*, DAO (Dis. Aquat. Org.) 111 (2014) 119–127 https://doi.org/10.3354/dao02772.

[7] Y.Z. Sun, H.L. Yang, Z.C. Ling, J.B. Chang, J.D. Ye, Gut microbiota of fast and slow growing grouper *Epinephelus coioides*, African Journal of Microbiology Reseach 3 (11) (2009) 713–720.

[8] M. Sparo, G. Delpech, S. Batisttelli, J.Á. Basualdo, Immunomodulatory properties of cell wall extract from *Enterococcus faecalis*, cect7121, Braz. J. Infect. Dis. 18 (2014) 551–555 https://doi.org/10.1016/j.bjid.2014.05.005.

[9] P.J. Tian, B.L. Li, Y.J. Shan, J.N. Zhang, J.Y. Chen, M. Yu, et al., Extraction of peptidoglycan from *L. paracasei* subp. *Paracasei X*12 and its preliminary mechanisms of inducing immunogenic cell death in HT-29 cells, Int. J. Mol. Sci. 16 (2015) 20033–20049 https://doi.org/10.3390/ijms160820033.

[10] S. Morath, A. Geyer, T. Hartung, Structure–function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*, J. Exp. Med. 193 (2001) 393–397 https://doi.org/10.1084/jem.193.3.393.

[11] Y.Z. Sun, H.L. Yang, R.L. Ma, W.Y. Lin, Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*, Fish Shellfish Immunol. 29 (2010) 803–809 https://doi.org/10.1016/j.fsi.2010.07.018.

[12] Y.Z. Sun, H.L. Yang, R.L. Ma, K. Song, W.Y. Lin, Molecular analysis of autochthonous microbiota along the digestive tract of juvenile grouper *Epinephelus coioides* following probiotic *Bacillus pumilus* administration, J. Appl. Microbiol. 110 (2011) 1093–1103 https://doi.org/10.1111/j.1365-2672.2011.04967.x.

[13] T. Magoc, S.L. Salzberg, Flash: fast length adjustment of short reads to improve genome assemblies, Bioinformatics 27 (2011) 2957–2963 https://doi.org/10.1093/bioinformatics/btr507.

[14] R.C. Edgar, Search and clustering orders of magnitude faster than blast, Bioinformatics 26 (2010) 2460–2461 https://doi.org/10.1093/bioinformatics/btq461.

[15] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, et al., QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (2010) 335–336 https://doi.org/10.1038/nmeth.f.303.

[16] J.R. White, N. Nagarajan, M. Pop, Statistical methods for detecting differentially abundant features in clinical metagenomic samples, PLoS Comput. Biol. 5 (2009) e1000352https://doi.org/10.1371/journal.pcbi.1000352.

[17] Y.Z. Sun, H.Q. Xia, H.L. Yang, Y.L. Wang, W.C. Zou, TLR2 signaling may play a key role in the probiotic modulation of intestinal microbiota in grouper *Epinephelus coioides*, Aquaculture 430 (2014) 50–56 https://doi.org/10.1016/j.aquaculture.2014.03.042.

[18] M. Guo, J. Wei, X. Huang, Y. Huang, Q. Qin, Antiviral effects of β-defensin derived from orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 32 (2012) 828–838 https://doi.org/10.1016/j.fsi.2012.02.005.

[19] J. Wei, D. Xu, J. Zhou, H. Cui, Y. Yan, Z. Ouyang, et al., Molecular cloning, characterization and expression analysis of a C-type lectin (Ec-CTL) in orange-spotted grouper, *Epinephelus coioides*, Fish Shellfish Immunol. 28 (2010) 178–186 https://doi.org/10.1016/j.fsi.2009.10.020.

[20] B.E.V. Koch, S. Yang, G. Lamers, J. Stougaard, H.P. Spaink, Intestinal microbiome adjusts the innate immune setpoint during colonization through negative regulation of MyD88, Nat. Commun. 9 (2018) 4099 https://doi.org/10.1038/s41467-018-06658-4.

[21] S.T. Tapia-Paniagua, M. Chabrillón, P. Díaz-Rosales, I.G. de la Banda, C. Lobo, M.C. Balebona, M.A. Moriñigo, Intestinal microbiota diversity of the flat fish *Solea senegalensis* (Kaup, 1858) following probiotic administration, Microb. Ecol. 60 (2010) 310–319 https://doi.org/10.1007/s00248-010-9680-z.

[22] R.M.W. Ferguson, D.L. Merrifield, G.M. Harper, M.D. Rawling, S. Mustafa, S. Picchietti, J.L. Balcázar, S.J. Davies, The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*), J. Appl. Microbiol. 109 (2010) 851–862 https://doi.org/10.1111/j.1365-2672.2010.04713.x.

[23] W. Liu, P. Ren, S. He, L. Xu, Y. Yang, Z. Gu, Z. Zhou, Comparison of adhesive gut bacteria composition, immunity, and disease resistance in juvenile hybrid tilapia fed two different *Lactobacillus* strains, Fish Shellfish Immunol. 35 (2013) 54–62 https://doi.org/10.1016/j.fsi.2013.04.010.

[24] D.L. Merrifield, J.L. Balcazar, C.L. Daniels, Z. Zhou, O. Carnevali, Y.Z. Sun, S.H. Hoseinifar, E. Ringø, Indigenous lactic acid bacteria in fish and crustaceans, in: D.L. Merrifield, E. Ringø (Eds.), Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics, Wiley-Blackwell, West Sussex, 2014, pp. 128–168 https://doi.org/10.1002/9781118897263.

[25] E. Ringø, S.H. Hoseinifar, K. Ghosh, H.V. Doan, B.R. Beck, S. Song, Lactic acid bacteria in finfish-an update, Front. Microbiol. 9 (2018) 1818 https://doi.org/10.3389/fmicb.2018.01818.

[26] A.E. Toranzo, B. Magariños, J.L. Romalde, A review of the main bacterial fish diseases in mariculture systems, Aquaculture 246 (2005) 37–61 https://doi.org/10.1016/j.aquaculture.2005.01.002.

[27] I.M. Moi, N.N. Roslan, A.T.C. Leow, M.S.M. Ali, R.N.Z.R.A. Rahman, A. Rahimpour, et al., The biology and the importance of photobacterium, species, Appl. Microbiol. Biotechnol. 101 (2017) 4371–4385 https://doi.org/10.1007/s00253-017-8300-y.

[28] F.S. de Medina, M. Ortega-González, R. González-Pérez, F. Capitán-Cañadas, O. Martínez-Augustin, Host-microbe interactions: the difficult yet peaceful coexistence of the microbiota and the intestinal mucosa, Br. J. Nutr. 109 (2013) S12–S20 https://doi.org/10.1017/S0007114512004035.

[29] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (2013) 1729–1739 https://doi.org/10.1016/j.fsi.2013.09.032.

[30] E. Ringø, Z. Zhou, J.L.G. Vecino, S. Wadsworth, J. Romero, Å. Krogdal, R.E. Olsen, A. Dimitroglou, A. Foey, S. Davies, M. Owen, H.L. Lauzon, L.L. Martinsen, P. DeSchryver, P. Bossier, S. Sperstad, D.L. Merrifield, Effect of dietary components on the gut microbiota of aquatic animals. A never ending story? Aquacult. Nutr. 22 (2015) 219–282 https://doi.org/10.111/anu.1236.

[31] D.C. Broekman, G.H. Guðmundsson, V.H. Maier, Differential regulation of cathelicidin in salmon and cod, Fish Shellfish Immunol. 35 (2013) 532–538 https://doi.org/10.1016/j.fsi.2013.05.005.