



Commensal *Bacillus pumilus* SE5-Derived Peptidoglycan and Lipoteichoic Acid Showed Synergistic Effects in Improving Growth, Immunity, and Intestinal Health of Grouper (*Epinephelus coioides*)

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Accepted: 13 May 2024

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Abstract

Commensal-derived peptidoglycan (PG) or lipoteichoic acid (LTA) can improve the growth, immunity, and intestinal health of fish, but it is not clear whether the two components have synergistic effects. To clarify this, grouper (*Epinephelus coioides*) was fed basal diet (CG) or diets containing 1.0×10^8 CFU/g heat-inactivated SE5 (HIB), PG (21.30 mg/kg), LTA (6.70 mg/kg), mixture (PL1) of PG (10.65 mg/kg) and LTA (3.35 mg/kg), and mixture (PL2) of PG (21.30 mg/kg) and LTA (6.70 mg/kg). Improved growth performance and feed utilization were observed in groups PG, LTA, PL1, and PL2, and the optimum growth performance was recorded in group PL1. Furthermore, improved serum alkaline phosphatase (AKP) activity and immunoglobulin M (IgM) and complement C3 (C3) contents were observed in all treatments, and the AKP activity in group PL1 was significantly superior to that of groups PG and LTA. Although PG and LTA alone or in combination exert comparable effects on intestinal microbiota and physical structure, obviously enhanced intestinal protease activity was observed in group PL1. The combined efficacy of PL1 could further potentiate the immune response by modulating the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and upregulating the expression of antimicrobial peptides (epinecidin-1, hepcidin-1, and β -defensin) as well as IgM. At the same time, group PL1 could further mitigate intestinal inflammation by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory cytokines. In conclusion, probiotic *B. pumilus* SE5-derived PG and LTA mixture (10.65 mg/kg PG and 3.35 mg/kg LTA) exhibits better potential for improving the growth performance, intestinal health, and immune function compared to another mixture (21.30 mg/kg PG and 6.70 mg/kg LTA) and PG or LTA alone in grouper.

Keywords *Bacillus pumilus* · Peptidoglycan · Lipoteichoic acid · Immunity · Intestinal health · *Epinephelus coioides*

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Introduction

The enormous amount utilization of antibiotics leads to heightened possibilities for the existence of residual antibiotics in fishery commodities [1–3]. Consequently, the conventional aquaculture approach reliant on antibiotics is poised to be eradicated as the contemporary aquatic industry progresses. Probiotics have been chosen and tapped as the preferred eco-friendly prophylactic approach as an alternative to antibiotics for the improvement of disease control and aquatic production [4–6]. However, the challenge of active quality control during processing, transportation, and preservation of probiotics has emerged as a major obstacle to their development [7].

Postbiotics, defined as “reagent of inanimate microorganisms and/or their components that is beneficial to the health of host” by The International Scientific Association

for Probiotics and Prebiotics, receive extensive attention recently [8]. Postbiotics have been found to play a crucial role in preserving intestinal homeostasis, mitigating intestinal inflammation, and boosting immunity in aquatic animals [9]. Following the dietary administration of peptidoglycan in *Oncorhynchus mykiss*, *Paralichthys olivaceus*, and *Apostichopus japonicus* [10–12] and lipoteichoic acid in *Epinephelus coioides* and *Pampus argenteus*, enhanced immune function of these reagents has been demonstrated [13]. It is generally acknowledged that the microbial-associated molecular patterns (MAMPs), such as peptidoglycan and lipoteichoic acid from commensal microorganisms/probiotics, can be recognized by pattern recognition receptor (PPR), which would facilitate to maintain the homeostasis of commensal microbial community while limiting the invasion of pathogenic bacteria [14].

Bacillus pumilus SE5, which was isolated from the intestine of the grouper, showed impressively potentials in improving growth performance, feed utilization, regulating the microbiota homeostasis, and maintaining fish health [15]. In addition, dietary administration of *B. pumilus* SE5-derived peptidoglycan (PG) and lipoteichoic acid (LTA) alone can effectively improve the growth performance, nutrient utilization, intestinal immunity, and microbial balance of the grouper [16]. However, it is unclear whether the two cellular components (PG and LTA) have synergistic effects in improving the growth and health of fish. Therefore, we evaluated the effects of PG and LTA alone or in combination on the growth, immunity, and intestinal health in *E. coioides*.

Materials and Methods

Animal Ethics Statement

The experimental design and procedures in this study were reviewed and approved by the Animal Ethics Committee of Jimei University, Xiamen, China (approval number: 2011–58).

Probiotic Strain and Cell Wall Component Preparation

Probiotic *B. pumilus* SE5, which was isolated from the intestine of juvenile *E. coioides* [17], was prepared and cultured as previously described [18]. The 95 °C water bath was used to inactivate the live bacterial suspension after the re-suspended cells in PBS were harvested and counted by plating on tryptone soya agar (TSA).

A TCA-based purification method was used to extract PG from *B. pumilus* SE5 as previously described with slightly modification [18]. Briefly, cell sediment was suspended in TCA and treated with a solvent, chloroform, and methanol. The undissolved material was agitated in Tris–HCL

containing trypsin. Finally, the sediment was lyophilized after washing in sterile water.

LTA was extracted from *B. pumilus* SE5 as previously described [18]. Briefly, the suspension of bacteria was mixed with n-butanoland; then, the sediment was lyophilized followed by being mixed with chromatography buffer. The target product was obtained by chromatography with octyl agarose and confirmed as previously described [19].

Diet Preparation and Experimental Design

The ingredients listed in Table 1 was used to formulate the basal diet, and its proximate composition was determined in accordance with the protocols of AOAC (2005). Samples were burned in a muffle furnace to a constant weight, and the ash composition was calculated. The crude protein content was determined by Kjeldahl ($N \times 6.25$) (Kjeltec 2200, FOSS, Denmark). The crude lipid content was determined based on the chloroform–methanol extraction method [20]. A generally recognized dose (1.0×10^8 CFU/g) of *B. pumilus* SE5 has verified the potential in improving the feed efficiency and immune response of *E. coioides* [21, 22]. The heat-inactivated SE5 with the dose of 1.0×10^8 CFU/g, the PG (21.30 mg/kg, extracted from 1.0×10^8 CFU/g SE5), LTA (6.70 mg/kg, extracted from 1.0×10^8 CFU/g SE5), the mixture of PG (10.65 mg/kg) and LTA (3.35 mg/kg), and the mixture of PG (21.30 mg/kg) and LTA (6.70 mg/kg) were supplemented to the basal diet (CG), which was named as HIB, PG, LTA, PL1, and PL2, respectively. The heat-inactivated SE5, PG, and LTA were added to diets with demand doses followed by being mixed in a dimensional drum mixer (SYH-100, Punaier Drying Equipment Co., Ltd., Changzhou, China) and then extruded to produce 5-mm pellets (CD4XITS extruder, South China University of Technology, Guangzhou, China).

The animal trial was carried out in Haikang Aquaculture Research Base of Dabeinong group (Zhaoan, China). The feeding experiment was carried out in 18 recirculating tanks that held 450-L saltwater with a salinity of 30 g/L at 26 ± 2 °C. Thirty fish (14.69 ± 0.05 g) were randomly deposited into each tank. Fish in triplicate tanks fed to apparent satiation twice daily (08:30 and 18:30) for 60 days. After 24 h of fasting, fish were anesthetized with diluted eugenol (1:10,000) for sampling. To access the survival rate and growth performance (final weight, weight gain rate, specific growth rate, feed intake, and feed conversion rate) [16], fish number and weight in each tank were recorded, and then, the blood of caudal vein was collected. Thereafter, the fish was dissected under sterile conditions and tissue samples were collected, and visceral index (VSI), hepatic index (HSI), intestinal somatic index (ISI), and intestinal length index (ILI) were determined. Four intestinal samples per treatment were

Table 1 Formulation and proximate composition of the experimental diets (g/100 g dry matter)

Ingredients	
Domestic fish meal ^a	20.00
White fish meal ^a	26.00
Soybean meal ^a	20.00
Wheat gluten ^a	6.00
Wheat flour ^a	10.40
Chicken meal ^a	5.00
Squid paste ^a	3.00
Fish oil ^a	3.00
Soybean oil ^a	2.00
Lecithin ^a	1.00
Vitamin C ^a	0.10
Ca(H ₂ PO ₄) ₂ ^a	2.00
Choline chloride ^a	0.50
Mineral premix ^b	0.60
Vitamin premix ^c	0.40
Proximate composition	
Crude protein	48.80
Crude lipid	12.38
Ash	12.32

^aSupplied by Xiamen Jiakang Aquatic Feed Co., Ltd. (Fujian, China)

^bVitamin premix containing the following (mg/kg diet): thiamin, 10; riboflavin, 8; pyridoxine HCl, 10; vitamin B12, 0.2; vitamin K3, 10; inositol, 100; pantothenic acid, 20; niacin acid, 50; folic acid, 2; biotin, 2; retinol acetate, 400; cholecalciferol, 5; alpha-tocopherol, 100; ethoxyquin, 150; wheat middling 132.8

^cMineral premix containing the following (mg/kg diet): KCl, 200; KI, 60; CoSO₄, 100; CuSO₄·5H₂O, 24; FeSO₄·H₂O, 400; ZnSO₄·H₂O, 174; MnSO₄·H₂O, 78; MgSO₄·7H₂O, 800; Na₂SeO₃, 50; zeolite, 311.4

stored in −80 °C refrigerator for microbial analysis, and the remaining samples were kept in −80 °C refrigerator for digestive enzyme activity and gene expression analysis.

Digestive Enzymatic Analysis

Digestive amylase, trypsin, and lipase activities of the total intestine ($n = 5$) from each group was analyzed using corresponding kits (BioVision, USA) according to the manufacturer's instructions.

Hematological Analysis

The kits of Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used to test the serum protein concentration, glutamate–oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), and alkaline phosphatase (AKP) activities. The serum complements 3 (C3) and immunoglobulin M (IgM) concentrations were determined using the enzyme-linked immunosorbent assay kits (Shanghai Jianglei Biotechnology Co., Ltd., Shanghai, China).

Intestinal Morphology Analysis

Tissues were stained with hematoxylin and eosin (HE) staining to assess morphological of the intestine samples [23]. The foregut samples were treated with ethanol and xylene treatment followed by embedded in paraffin to cutting into a 6-μm slice and then stained with HE. The morphology of the intestine was micrographed using a Leica DM5500B microscope (Germany). Measurement of muscle thickness (MT) and mucosal folding height (MFH) was accomplished by Lecia Application Suite version 4.7.0 (Leica Microsystems, Germany).

Intestinal Microbiota Analysis

Total DNA of intestinal samples was extracted and checked; then, the V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction [24]. The Illumina HiSeq platform (Beijing Biomarker Biotechnology Co., Ltd., Beijing, China) was used to perform high-throughput sequencing. Gut microbiota abundance and diversity were analyzed using BMKCloud1 as reported in our previous study [18].

Intestinal Gene Expression Analysis

Total RNA of the intestinal samples was extracted as described previously [21, 25]. Intestinal barrier-related genes (Claudin3, Caspase8, Caspase9, Occludin, and ZO-1) and immune-related genes including pattern recognition receptors (TLR1, TLR2, TLR3, TLR5, TLR22, and NOD2), *NF-κB* pathway-related genes (MyD88, TAK1, IKKα, IKKβ, NF-κB, BCL2, and BCLXL), *MAPK* pathway-related genes (ERK1, ERK2, MKK4, MKK6, and P38), antibacterial peptides (hepcidin-1, β-defensin, and epinecidin-1), inflammatory cytokine genes (IL-8, IL-12, IL-1β, TNFα, IL-10, and TGF-β1), and immune effector genes (IgM, CD4, and MHCIIα) were determined using RT-qPCR with specific primers (Table 2). The ABI 7500 real-time PCR Detection system (Applied Biosystems, California, USA) was used to perform RT-qPCR in a 20-μL reaction system utilizing SYBR®

Table 2 Real-time PCR primers used for intestinal genes of *E. colioides*

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>β-Actin</i>	GATCTGGCATCACACCTTCT	CATCTTCTCCCTGTTGGCTT
<i>Occludin</i>	GGCTACGGTGATCGTGTGTGT	CCGCCTCCATAACCTCCTCCAT
<i>Claudin3</i>	GCATTGACGACGAGGCATCCAA	GCCGACCAGGAGACAGGAATGA
<i>ZO-1</i>	CGGCAGATCAGCAATGGCAACC	TGGTTCAGGCAGCGGAGGTAAC
<i>IL-1β</i>	GCAACTCCACCGACTGATGA	ACCAGGCTGTTATTGACCCG
<i>TNF-α</i>	GGATCTGGCGCTACTCAGAC	CGCCCAGATAAATGGCGTTG
<i>IL-8</i>	AAGTTTGCCTTGACCCGAA	TGAAGCAGATCTCTCCCGGT
<i>IL-12</i>	CCAGATTGCACAGCTCAGGA	CCGGACACAGATGGCCTTAG
<i>IL-10</i>	GTCCACCAGCATGACTCCTC	AGGGAAACCCTCCACGAATC
<i>TGF-β1</i>	GCTTACGTGGGTGCAAACAG	ACCATCTCTAGGTCCAGCGT
<i>IgM</i>	ACCGTGACCCTGACTTGCTATG	CCCAGTGGACCTGACAATAGC
<i>MHC-II</i>	ATGAATGCCTTGCCTGAG	CTGCTGGGCCTTGTAGTT
<i>CD4</i>	TGGACTGATGGCAATGAACTGA	GCAGCGGAGTGGATGGTTTC
<i>Hepcidin</i>	TGCTCGCCTTCATTTGC	GTCGGGTAGCAGTAAGGAG
<i>Epinecidin</i>	ATCGCCCTCTTCTTGTGTTGTCG	GCAGCTCTTCCACGCCATGTC
<i>β-Defensin</i>	ATGCTGTGCTGTGCGGTCTTAG	GCGTGTTAGAAGTCCAAGAGGTGTC
<i>TLR1</i>	CCAGGGTCGCAGAGTCCTATC	GCCAGCCAAGTTCAGTTTCGT
<i>TLR2</i>	AGGGTTCAGAAGGGTTGCTAT	CAGGAAGGAAGTCCCCTTTGT
<i>TLR5</i>	CTGACCCTGATGCTTTTCG	GCTACTTTACTGCTGTGTG
<i>TLR22</i>	TCGTGTTTATGGTGGCA	GTGGGTGTTGTAGGAGATG
<i>NOD2</i>	GGGGCAACAGGATAGGTGA	TTGATAATGTTGGCGAGGG
<i>MyD88</i>	GCATTGACGACGAGGCATCCAA	GCCGACCAGGAGACAGGAATGA
<i>TAK1</i>	TCTCAAGGGAGCAACGACAC	GCAGGCAGACTCTCAACACT
<i>IKKα</i>	TGGCTGAGAGCGAACAAGTCCT	AGCAGAGGCGGCACTGAAGAT
<i>IKKβ</i>	CTGAGAGCGAACAAGTCCTGATGAG	GCAGAGGCGGCACTGAAGATG
<i>NF-κB</i>	CTTACATTCCGCCCTCAGT	TGCAACAACGCCTTCAAACC
<i>Bcl-2</i>	GTGCGTGGAGTGCCTTGAGAA	CGTCCCATCCTCTTTGGCTCT
<i>Bcl-xL</i>	AGTAACGGCTTGCTGGTCAA	GCTGTGGTAGGCTGTGTCA
<i>P38</i>	AGCGGAGCCTTACGACCAGAG	GTGCCTCCTCCTCCTCCTCAG
<i>ERK1</i>	GACAAGCCAGCGTGTAGCCATC	AATGTGCCGTGCCCTGAGAATG
<i>ERK2</i>	ATCCTACGAGGGCTGAAGTACATCC	GGTGGTGTGAGCAGAAGGTTGG
<i>MKK4</i>	GTGGATGAGAAGGAGCAGAAGCAG	CGCCGTAGAACTGAACAATGTAGGG
<i>MKK6</i>	GACTTCGGCATCAGCGGCTAC	CCTTCTGGTTCGTCTCTGGGTTG

ZO-1 Zonula occludens protein 1, *IL-1β* interleukin-1β, *TNF-α* tumor necrosis factor-α, *IL-8* interleukin-8, *IL-12* interleukin-12, *IL-10* interleukin-10; *TGF-β1* transforming growth factor β 1, *IgM* immunoglobulin M, *MHC-II* major histocompatibility complex class II, *CD4* cluster of differentiation 4, *TLR1* toll-like receptor 1, *TLR2* toll-like receptor 2, *TLR5* toll-like receptor 5, *TLR22* toll-like receptor 22, *NOD2* nucleotide-binding oligomerization domain 2, *MyD88* myeloid differentiation factor 88, *TAK1* transforming growth factor-β-activated kinase 1, *IKKα* inhibitor of κB kinase α, *IKKβ* inhibitor of κB kinase β, *NF-κB* nuclear factor kappa-B, *Bcl-2* B-cell lymphoma-2, *Bcl-xL* B-cell lymphoma-extra large, *ERK1* extracellular regulated protein kinases 1, *ERK2* extracellular regulated protein kinases 1, *MKK4* mitogen-activated protein kinase kinase4, *MKK6* mitogen-activated protein kinase kinase4

Table 3 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on growth performance, feed utilization and body index of *E. coioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
FBW (g)	50.59 ± 2.08 ^b	60.16 ± 2.72 ^a	61.47 ± 2.04 ^a	60.59 ± 3.00 ^a	62.37 ± 2.31 ^a	60.84 ± 2.55 ^a	0.01
WGR (%)	244.38 ± 14.17 ^b	309.56 ± 18.51 ^a	318.41 ± 13.91 ^a	312.46 ± 20.43 ^a	324.60 ± 15.75 ^a	314.16 ± 17.35 ^a	0.01
SGR (%/d)	2.18 ± 0.07 ^b	2.48 ± 0.08 ^a	2.54 ± 0.06 ^a	2.49 ± 0.09 ^a	2.56 ± 0.07 ^a	2.51 ± 0.08 ^a	<0.01
FI (g/fish)	39.51 ± 3.23 ^{ab}	50.22 ± 1.13 ^c	37.37 ± 1.94 ^a	44.89 ± 1.51 ^{bc}	41.26 ± 2.13 ^{ab}	40.92 ± 3.08 ^{ab}	<0.01
FCR	1.10 ± 0.08 ^c	1.10 ± 0.02 ^c	0.80 ± 0.04 ^a	1.00 ± 0.03 ^{bc}	0.87 ± 0.04 ^{ab}	0.89 ± 0.67 ^{ab}	<0.01
SR (%)	83.33 ± 0.00 ^b	86.67 ± 3.34 ^b	100 ± 0.00 ^a	81.66 ± 1.66 ^b	95.00 ± 1.67 ^a	97.78 ± 1.11 ^a	0.03
CF	2.74 ± 0.05 ^{ab}	2.91 ± 0.05 ^a	2.71 ± 0.05 ^{ab}	2.80 ± 0.05 ^{ab}	2.76 ± 0.05 ^{ab}	2.66 ± 0.10 ^b	0.10
VSI	8.45 ± 0.45	8.50 ± 0.34	8.81 ± 0.33	9.24 ± 0.28	9.28 ± 0.25	8.57 ± 0.28	0.28
HSI	2.42 ± 0.97	2.28 ± 0.65	2.08 ± 0.66	2.46 ± 0.96	2.40 ± 0.55	2.03 ± 0.75	0.35
ISI	1.16 ± 0.09 ^a	1.08 ± 0.06 ^{ab}	1.21 ± 0.05 ^a	0.93 ± 0.04 ^{bc}	0.89 ± 0.04 ^c	0.93 ± 0.06 ^{bc}	<0.01
ILI	114.62 ± 4.33 ^c	118.26 ± 2.93 ^{bc}	126.66 ± 3.06 ^{ab}	133.77 ± 3.86 ^a	121.85 ± 3.80 ^{bc}	120.56 ± 3.96 ^{bc}	<0.01

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA, FBW final body weight, WGR weight gain rate, SGR specific growth rate, FI feed intake, FCR feed conversion rate, SR survival rate, CF condition factor, VSI viscerosomatic index, HSI hepatosomatic index, ISI intestosomatic index, ILI intestinal length index

Green Pro Taq HS. β -Actin was used as the housekeeping gene. The gene expression levels were calculated using $2^{-\Delta\Delta CT}$ method.

Data Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Data were examined by one-way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS), release 19.0 (SPSS Inc., Chicago, IL, USA). Significant differences were indicated at $P \leq 0.05$.

Results

Growth Performance

The significant increment of final weight, weight gain rate, and specific growth rate was observed in treatments as compared

with the control ($P = 0.01$, $P = 0.01$, and $P < 0.01$). The feed intake in groups PL1 and PL2 were slightly higher than that of control ($P > 0.05$), and the feed conversion rate in groups PG, PL1, and PL2 were reduced significantly ($P < 0.01$). The survival rate in the groups PG, PL1, and PL2 were significantly higher than that of the control ($P = 0.03$, Table 3).

The CF, HIS, and VSI were not impacted by the PG and LTA alone or in combination ($P = 0.10$, $P = 0.35$, and $P = 0.28$). The ISI in groups LTA, PL1, and PL2 were significantly lower than that of the control ($P < 0.01$).

Digestive Enzyme Activities

As shown in Table 4, the highest amylase activity was observed in group HIB, which was significantly higher than other groups ($P = 0.01$). The lipase activity in groups LTA and PL2 was significantly higher than that of groups HIB and control ($P < 0.01$). The increased protease activity was

Table 4 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on intestinal digestive enzyme activity of *E. coioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
Amylase (U/gprot)	0.52 ± 0.01 ^b	0.87 ± 0.07 ^a	0.55 ± 0.04 ^b	0.53 ± 0.04 ^b	0.47 ± 0.05 ^b	0.64 ± 0.07 ^b	0.01
Lipase (U/gprot)	0.46 ± 0.04 ^a	0.61 ± 0.04 ^{ab}	0.75 ± 0.04 ^{bc}	0.91 ± 0.08 ^c	0.72 ± 0.03 ^{bc}	0.92 ± 0.09 ^c	<0.01
Protease (U/gprot)	22.77 ± 1.62 ^a	47.45 ± 0.32 ^b	46.31 ± 2.91 ^b	60.19 ± 1.30 ^c	62.61 ± 1.39 ^c	46.09 ± 1.27 ^b	<0.01

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

Table 5 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on serum biochemical indices of *E. coioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
AKP (U/mgprot)	5.63 ± 0.26 ^a	7.51 ± 0.65 ^{bc}	6.59 ± 0.35 ^{abc}	5.88 ± 0.25 ^{ab}	8.00 ± 0.41 ^c	6.26 ± 0.31 ^{ab}	< 0.01
GOT (U/mgprot)	88.06 ± 6.23 ^c	25.05 ± 2.03 ^a	51.39 ± 2.88 ^b	25.06 ± 1.56 ^a	38.16 ± 1.78 ^{ab}	24.55 ± 2.95 ^a	< 0.01
GPT (U/mgprot)	101.38 ± 1.93 ^c	61.56 ± 3.76 ^a	79.77 ± 3.83 ^b	79.96 ± 4.13 ^b	58.59 ± 4.00 ^a	74.35 ± 4.29 ^{ab}	< 0.01
IgM (μg/mL)	183.06 ± 11.65 ^b	451.60 ± 27.12 ^a	461.92 ± 30.80 ^a	429.38 ± 21.24 ^a	527.91 ± 27.26 ^a	426.19 ± 31.55 ^a	< 0.01
C3 (μg/mL)	146.62 ± 2.99 ^a	186.84 ± 8.91 ^a	288.51 ± 15.15 ^b	284.65 ± 31.13 ^b	344.45 ± 21.17 ^b	328.33 ± 5.66 ^b	< 0.01

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA, AKP alkaline phosphatase, GOT glutamic oxaloacetic transaminase, GPT glutamic pyruvic transaminase, IgM immunoglobulin M, C3 complement C3

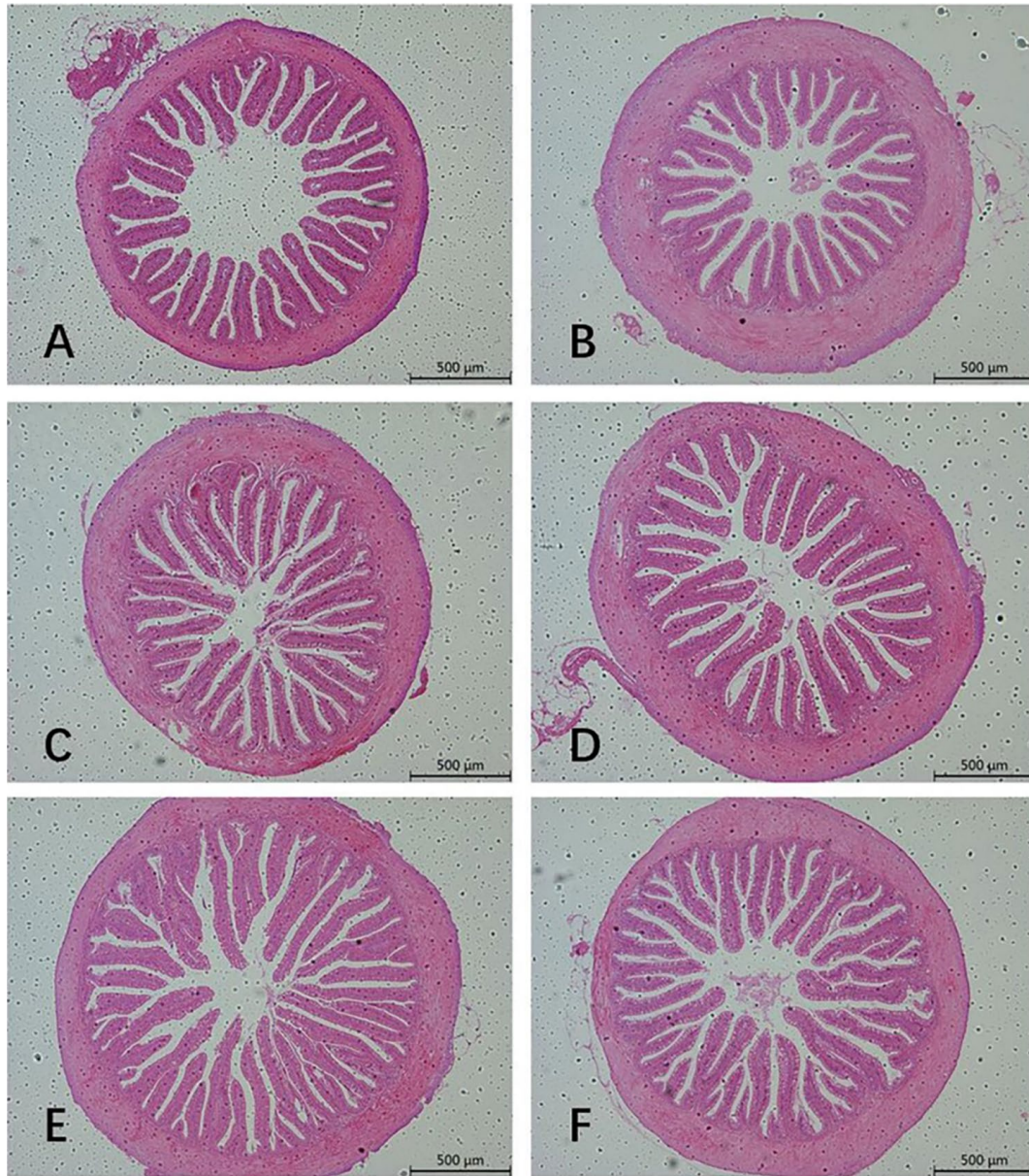


Fig. 1 The HE-stained section of intestinal tract of *E. coioides* (4x). Note: CG group (A), HIB=control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG;

LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

Table 6 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on intestinal morphology of *E. coioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
Villi length (μm)	434.92 \pm 5.69 ^a	440.16 \pm 5.60 ^a	574.73 \pm 6.42 ^c	573.39 \pm 4.67 ^c	649.66 \pm 1.66 ^d	487.20 \pm 4.90 ^b	< 0.01
Villi width (μm)	91.43 \pm 2.22 ^a	99.97 \pm 0.55 ^{bc}	109.64 \pm 0.33 ^d	106.07 \pm 1.31 ^{cd}	107.40 \pm 1.95 ^d	97.55 \pm 0.79 ^{ab}	< 0.01
Muscal thickness (μm)	101.83 \pm 2.35 ^a	154.66 \pm 5.05 ^b	164.12 \pm 2.75 ^{bc}	169.23 \pm 0.93 ^c	168.85 \pm 2.93 ^c	152.59 \pm 1.54 ^b	< 0.01
Goblet cell density (n/1000 μm)	61.77 \pm 1.14	63.52 \pm 2.16	64.27 \pm 2.55	64.17 \pm 0.87	68.74 \pm 1.59	67.08 \pm 1.35	0.22

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

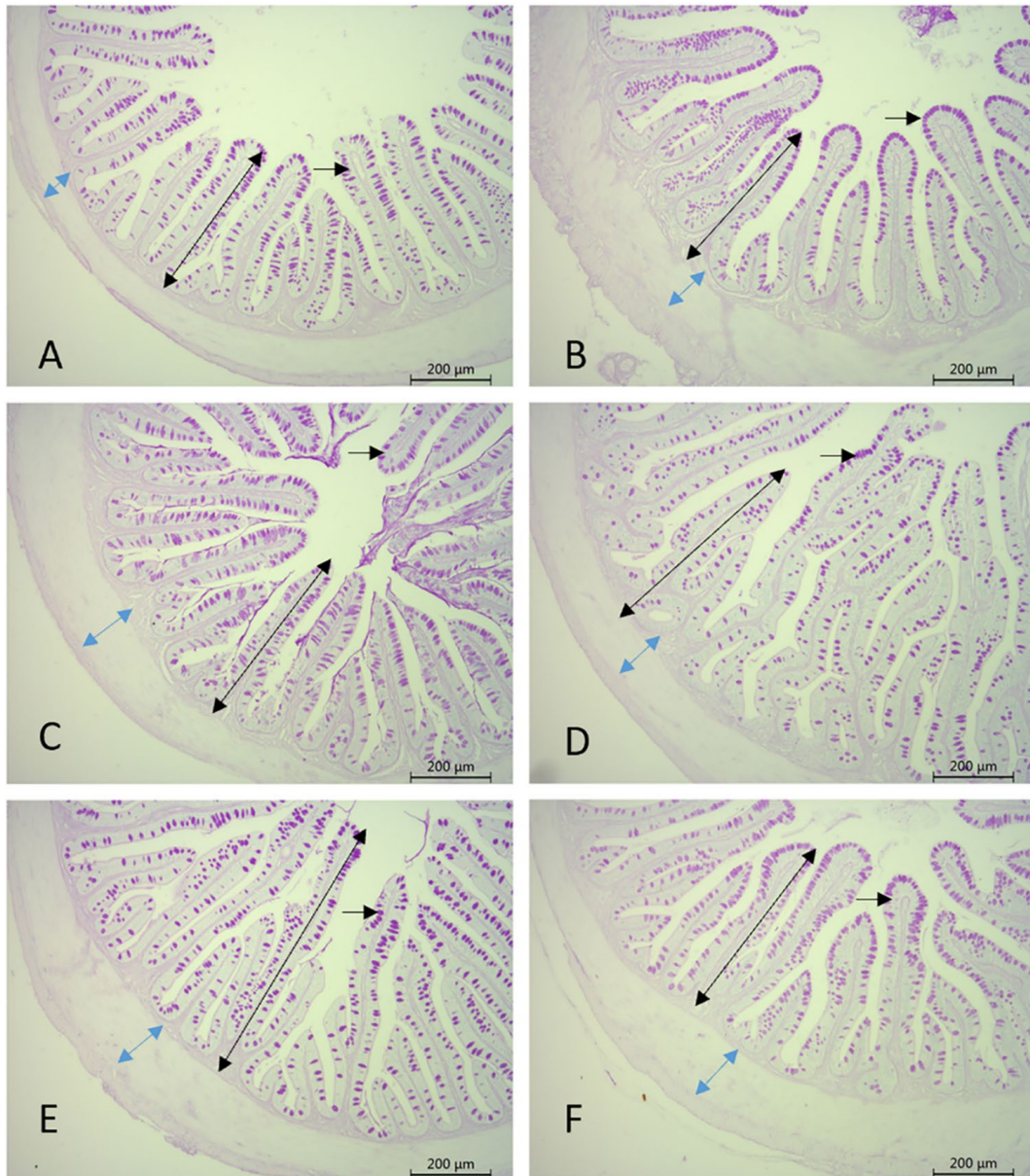


Fig. 2 The PAS-stained section of intestinal tract of *E. coioides* (10x). Blue bidirectional arrows indicate sarcomere thickness; black bidirectional arrows villi length; black unidirectional arrows indicate goblet cell after staining. Note: CG group (A), HIB group (B), PG group (C), LTA group (D), PL1 group (E), PL2 group (F).

CG=control group; HIB=control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

Table 7 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on alpha diversity of intestinal microbiota of *E. colioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
ACE	4154.59 ± 13.32	4263.92 ± 33.33	4163.16 ± 19.98	4204.46 ± 44.15	4263.39 ± 80.83	4240.12 ± 19.24	0.34
Chao1	4146.81 ± 29.29	4261.77 ± 33.06	4161.29 ± 19.99	4201.83 ± 44.06	4260.56 ± 80.67	4237.11 ± 19.11	0.74
Simpson	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.60
Shannon	9.84 ± 0.07	9.96 ± 0.04	9.89 ± 0.04	9.82 ± 0.03	9.91 ± 0.03	9.82 ± 0.03	0.88

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA, ACE abundance-based coverage estimator

observed in groups LTA and PL1 compared with groups CG, HIB, and PL2, and the increased protease activity was observed in group PL1 as compared with group PG ($P < 0.01$).

Hematological Analysis

Significantly higher alkaline phosphatase (AKP) activity was observed in group PL1 compared with group PG ($P < 0.01$).

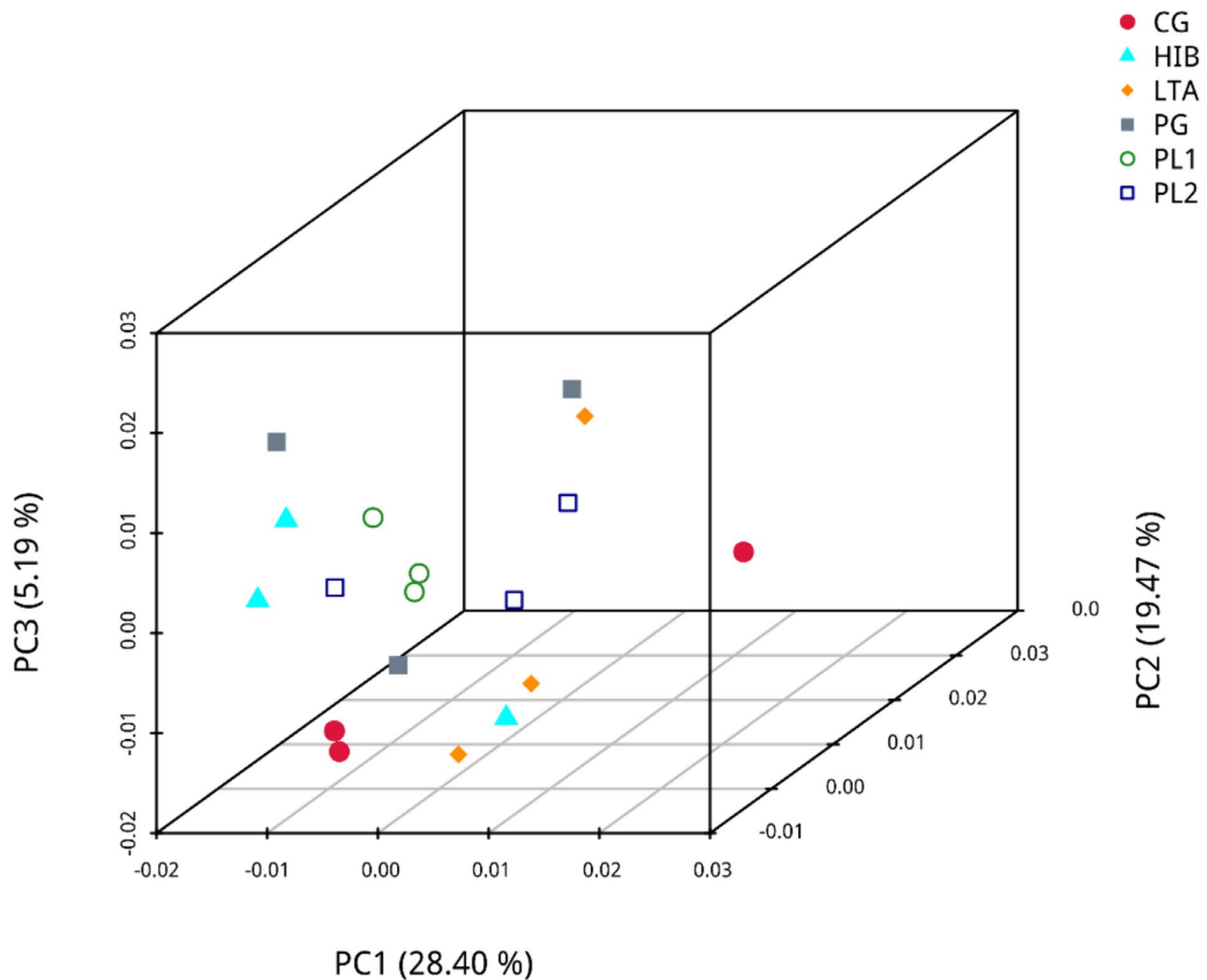


Fig. 3 Beta diversity of intestinal microbiota of *E. colioides*. CG=control group; HIB=control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG;

LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG

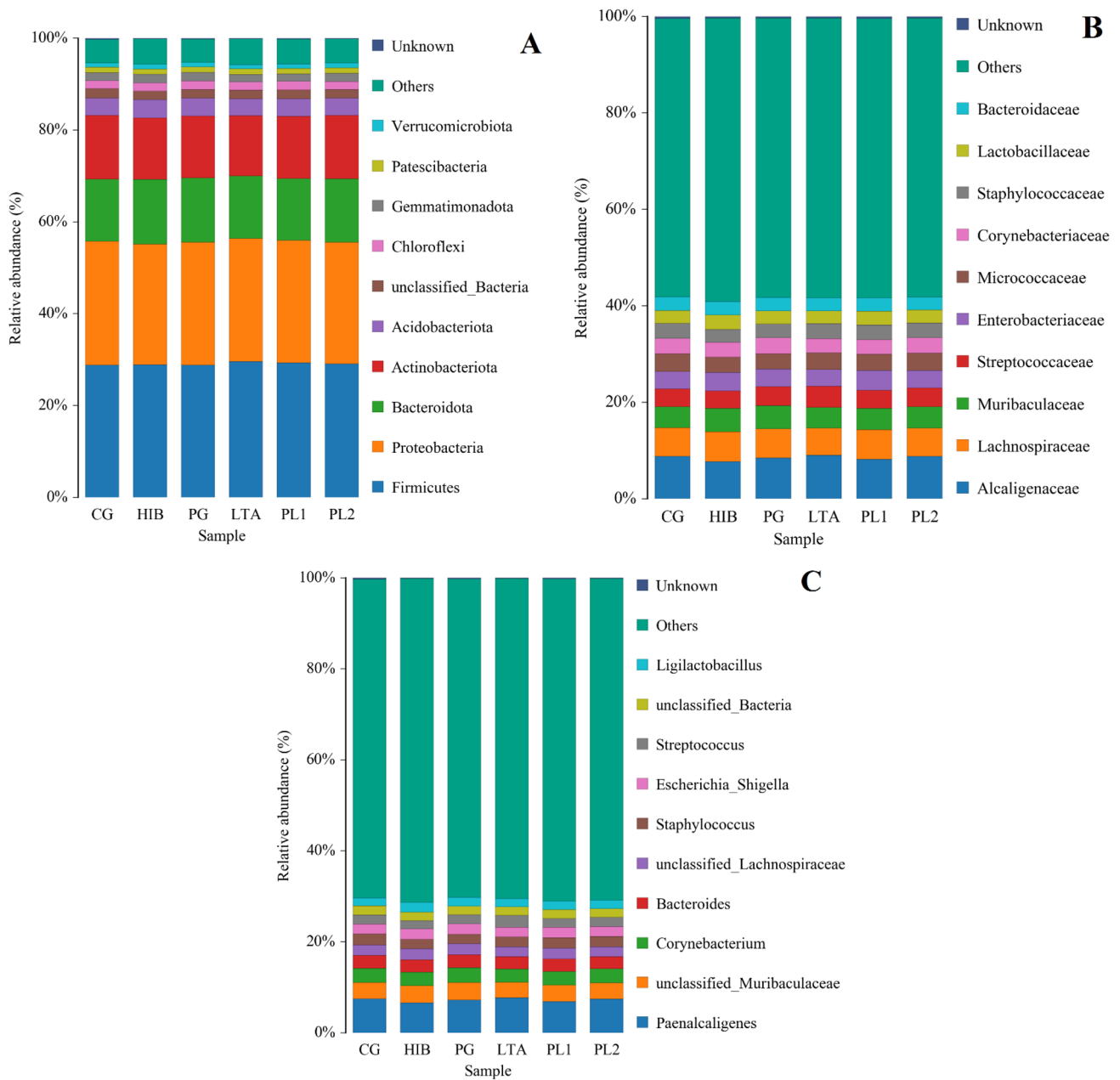


Fig. 4 Taxonomy classification of reads from 16S RNA V3–V4 regions of intestinal bacteria at phylum (A), family (B), and genus (C) taxonomic levels. Only top 15 most abundant phyla and genera (based on relative abundance) were shown in the figures. Other phyla and genera were all assigned as ‘Others’. CG=control group;

HIB=control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

The decreased glutamic oxaloacetic transaminase (GOT) activity in group PL2 was observed compared with group PG ($P < 0.01$). The immunoglobulin M (IgM) and complement C3 contents showed no variation among groups PG, LTA, PL1, and PL2, but significantly higher than the control ($P < 0.01$), and the maximum values were recorded in group PL1 (Table 5).

Intestinal Morphology

The improved villus length and width were observed in groups PG, LTA, and PL1 ($P < 0.01$, Fig. 1 and Table 6). Increased muscle thickness was observed in each treatment in comparison to the control ($P < 0.01$). The slightly

Table 8 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on relative abundance of intestinal bacteria at phylum level of *E. colioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
Firmicutes	28.83 ± 0.54	28.91 ± 0.61	28.82 ± 0.12	29.6 ± 0.32	29.32 ± 0.27	29.14 ± 0.25	0.29
Proteobacteria	26.94 ± 0.54	26.27 ± 0.85	26.77 ± 0.27	26.79 ± 0.42	26.66 ± 0.07	26.45 ± 0.41	0.99
Actinobacteriota	13.85 ± 0.87	13.34 ± 0.38	13.53 ± 0.41	13.18 ± 0.51	13.53 ± 0.29	13.83 ± 0.33	0.78
Bacteroidota	13.55 ± 0.64	14.09 ± 0.54	13.98 ± 0.36	13.59 ± 0.27	13.49 ± 0.09	13.78 ± 0.62	0.87
Acidobacteriota	3.80 ± 0.17	3.97 ± 0.10	3.83 ± 0.15	3.68 ± 0.20	3.82 ± 0.02	3.78 ± 0.09	0.76

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

Table 9 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on relative abundance of intestinal bacteria at subject level of *E. colioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
Alcaligenaceae	2.64 ± 0.17	3.03 ± 0.09	2.71 ± 0.06	2.65 ± 0.15	2.82 ± 0.16	2.72 ± 0.09	0.15
Lachnospiraceae	5.92 ± 0.40	6.17 ± 0.35	6.06 ± 0.23	5.61 ± 0.26	6.09 ± 0.17	5.84 ± 0.29	0.25
Muribaculaceae	4.40 ± 0.33	4.84 ± 0.15	4.80 ± 0.15	4.27 ± 0.06	4.48 ± 0.06	4.47 ± 0.20	0.18
Micrococcaceae	3.69 ± 0.44	3.22 ± 0.28	3.19 ± 0.27	3.45 ± 0.21	3.37 ± 0.09	3.56 ± 0.22	0.32
Streptococcaceae	3.65 ± 0.33	3.63 ± 0.26	3.92 ± 0.07	4.42 ± 0.74	3.73 ± 0.08	3.88 ± 0.23	0.34

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

increased number of microvilli goblet cells was observed in all treatments in comparison to the control ($P = 0.22$, Fig. 2).

Intestinal Microbiota

The PG and LTA treatments did not influence the OUT diversity indices (Shannon and Simpson) ($P = 0.88$ and $P = 0.60$) and the richness indexes (ACE and Chao1)

($P = 0.34$ and $P = 0.74$, Table 7). The slightly improved richness indexes (ACE and Chao1) were observed in groups PL1 and PL2 compared with groups PG and LTA. On the other hand, the three-dimensional analysis of principal component analysis (PCA) showed a considerable similarity between treatments and control (Fig. 3).

At phylum level, the most prominent bacterial phyla detected in all treatments were Firmicutes, Proteobacteria,

Table 10 Effects of *B. pumilus*-derived peptidoglycan and lipoteichoic acid on relative abundance of intestinal bacteria at genus level of *E. colioides*

Index	CG	HIB	PG	LTA	PL1	PL2	P-value
<i>Paenalcaligenes</i>	7.50 ± 0.99	6.60 ± 0.72	7.20 ± 0.64	7.73 ± 0.37	6.89 ± 0.35	7.41 ± 0.56	0.65
<i>Muribaculaceae</i>	3.42 ± 0.21	3.70 ± 0.26	3.82 ± 0.08	3.38 ± 0.03	3.61 ± 0.05	3.54 ± 0.12	0.33
<i>Corynebacterium</i>	2.83 ± 0.16	2.94 ± 0.06	3.37 ± 0.21	2.89 ± 0.06	3.03 ± 0.07	3.15 ± 0.17	0.11
<i>Bacteroides</i>	2.86 ± 0.19	2.73 ± 0.14	2.80 ± 0.10	2.72 ± 0.10	2.78 ± 0.02	2.59 ± 0.23	0.88
<i>Staphylococcus</i>	2.41 ± 0.26	2.05 ± 0.21	2.12 ± 0.24	2.28 ± 0.21	2.24 ± 0.05	2.28 ± 0.04	0.53
<i>Lachnospiraceae</i>	2.29 ± 0.17	2.43 ± 0.11	2.43 ± 0.06	2.14 ± 0.11	2.38 ± 0.14	2.20 ± 0.14	0.23
<i>Ligilactobacillus</i>	1.68 ± 0.04	1.89 ± 0.04	1.77 ± 0.09	1.74 ± 0.07	1.71 ± 0.09	1.81 ± 0.11	0.46
<i>Lactococcus</i>	1.63 ± 0.13	1.70 ± 0.08	1.75 ± 0.09	1.72 ± 0.08	1.80 ± 0.13	1.69 ± 0.09	0.90

CG control group, HIB control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5, PG control diet + 21.30 mg/kg PG, LTA control diet + 6.70 mg/kg LTA, PL1 control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA, PL2 control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA

Values in the same row with the same superscripts are not significantly different ($P > 0.05$)

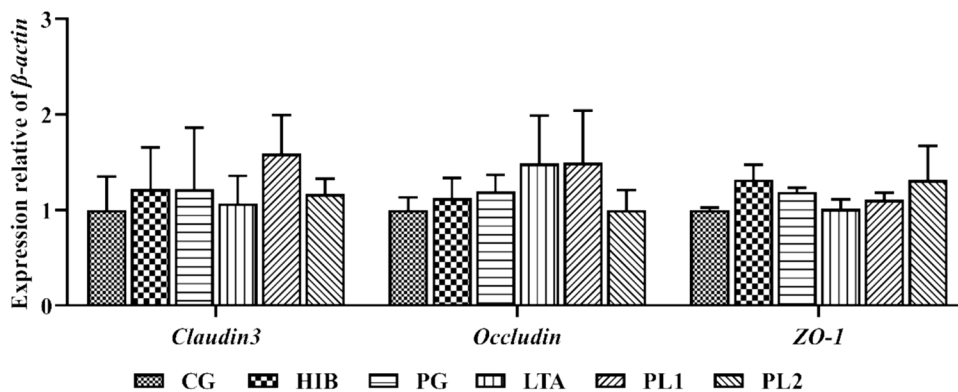


Fig. 5 The expression of *Claudin3*, *Occludin*, and *ZO-1* in the intestine of *E. coli*. *ZO-1*=zonula occludens protein 1; CG=control group; HIB=control diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=con-

trol diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

Bacteroidetes, and Actinobacteriota (Fig. 4). Slightly increased relative abundances of Firmicutes were observed in groups LTA, PL1, and PL2 compared with that of the control ($P = 0.29$), while slightly decreased Proteobacteria and Actinobacteriota ($P = 0.99$ and $P = 0.78$) in groups PG, LTA, PL1, and PL2 compared with those of the control (Table 8). There was no significant variation between all groups at subject level ($P > 0.05$, Table 9). At genus level, *Paenalcigenes*, *Muribaculaceae*, *Corynebacterium*, *Bacteroides*, and *Staphylococcus* constituted the most dominant genera. Although there was no significant alteration, relatively elevated *Ligilactobacillus* and *Lactococcus* abundances were observed in all treatments compared with the control ($P = 0.46$ and $P = 0.90$). Moreover, the relative abundances of *Muribaculaceae*, *Corynebacterium*, *Lachnospiraceae*, *Ligilactobacillus*, and *Lactococcus* in group PL1 were slightly higher than those of group LTA ($P > 0.05$, Table 10).

Relative Expression of Intestinal Physical Barrier-Related Genes

The PG and LTA alone or in combination led to a slight increment in the expression of intestinal Occludin, with the highest level observed in group PL1 ($P = 0.93$, Fig. 5). The expression of Claudin-3 and ZO-1 was slightly elevated in treatments ($P = 0.81$ and $P = 0.63$).

Relative Expression of Intestinal Immune-Related Genes

As shown in Fig. 6, the significant upregulation of intestinal TLR2 expression was observed in groups PL2 and PG compared to the control ($P = 0.01$). The expression of intestinal NOD2 in group PL1 was higher than that in groups PG and LTA and that in group PL2 was higher than that in group PG ($P < 0.01$). There was no significant difference in the

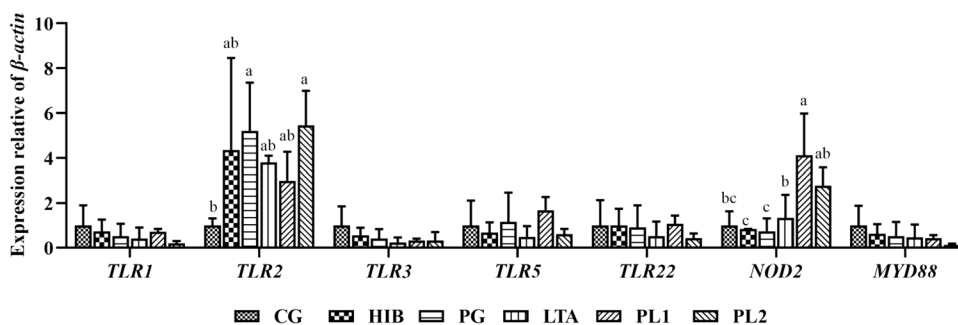


Fig. 6 The expression of TLRs (*TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR22*), *NOD2*, and *MYD88* in the intestine of *E. coli*. *TLR1*=toll-like receptor 1; *TLR2*=toll-like receptor 2; *TLR5*=toll-like receptor 5; *TLR22*=toll-like receptor 22; *NOD2*=nucleotide-binding oligomerization domain 2; *MyD88*=myeloid differentiation factor 88. CG=control group; HIB=control diet + 1.0×10^8 CFU/g heat-

inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

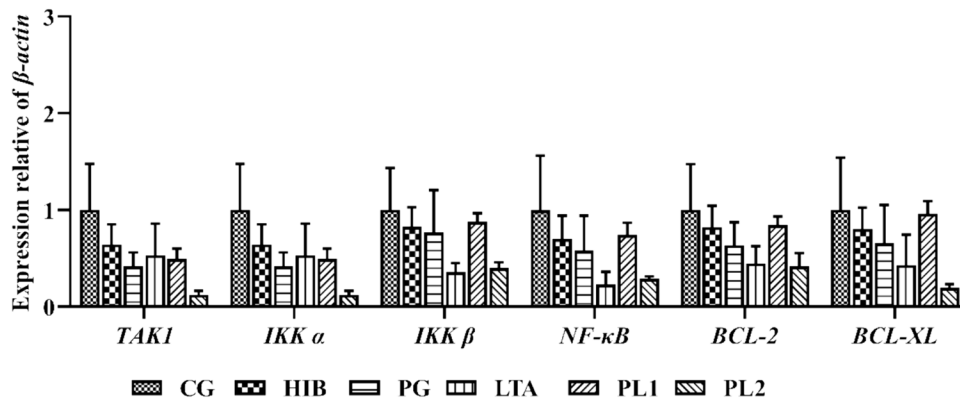


Fig. 7 The expression of *NF-κB* pathway (*TAK1*, *IKKα*, *IKKβ*, *NF-κB*, *BCL-2*, and *BCL-XL*) in the intestine of *E. colioides*. *TAK1*=transforming growth factor-β-activated kinase 1; *IKKα*=inhibitor of κB kinase α; *IKKβ*=inhibitor of κB kinase β; *NF-κB*=nuclear factor kappa-B; *Bcl-2*=B-cell lymphoma-2; *Bcl-xL*=B-cell lymphoma-extra large. CG=control group; HIB=control

diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

expression of other pattern recognition receptors (TLR1, TLR3, TLR5, and TLR22) among all groups ($P > 0.05$). The downregulated expression of the *NF-κB* pathway-related genes (*MyD88*, *TAK1*, *IKKα*, *IKKβ*, *NF-κB*, *BCL-2*, and *BCL-XL*) was observed in treatments ($P > 0.05$, Fig. 7). There was no significant difference in the *MAPK* pathway-related genes (*ERK2*, *MKK4*, *MKK6*, and *P38*) in all groups ($P > 0.05$, Fig. 8). The downregulated expression of *ERK1* was observed in groups LTA and PL2 ($P < 0.01$).

The upregulated expressions of intestinal antimicrobial peptides, which include β-defensin, epinecidin-1, and hepcidin-1, were observed in treatments (Fig. 9). The expressions of β-defensin and epinecidin-1 in group PL1 were significantly higher than those of groups PG and LTA ($P < 0.01$). The slightly upregulated expressions of the intestinal

immunity effectors, such as IgM, CD4, and MHCIIα, were observed in treatments ($P > 0.05$).

No significant variation in the pro-inflammatory cytokine gene expression was observed among groups PG, LTA, PL1, and PL2 ($P > 0.05$, Fig. 10). The upregulated expressions of IL-10 and TGF-β1 were observed in group PL1 compared with groups PG and LTA ($P < 0.01$ and $P = 0.04$).

Discussion

As the most critical MAMPs of Gram-positive bacteria, LTA and PG have been extensively reported to have positive functions in improving the growth and general health of aquatic animals [11]. However, no information is available

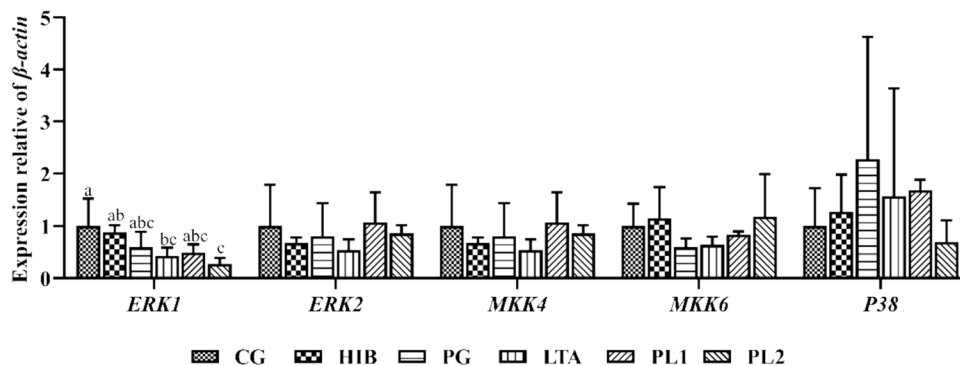


Fig. 8 The expression of *MAPK* pathway (*ERK1*, *MKK4*, *MKK6*, and *P38*) in the intestine of *E. colioides*. *ERK1*=extracellular regulated protein kinases 1; *ERK2*=extracellular regulated protein kinases 1; *MKK4*=mitogen-activated protein kinase kinase4; *MKK6*=mitogen-activated protein kinase kinase4. CG=control group; HIB=con-

trol diet + 1.0×10^8 CFU/g heat-inactivated *B. pumilus* SE5; PG=control diet + 21.30 mg/kg PG; LTA=control diet + 6.70 mg/kg LTA; PL1=control diet + 10.65 mg/kg PG + 3.35 mg/kg LTA; PL2=control diet + 21.30 mg/kg PG + 6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

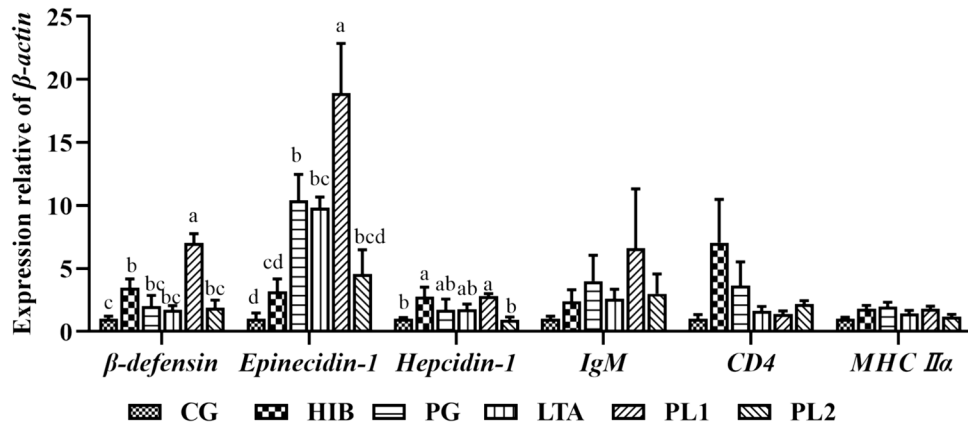


Fig. 9 The expression of antibacterial peptides (*Epinecidin-1*, *Hepsidin-1*, and β -defensin) and immunity effectors in the intestine of *E. coioides*. *IgM*=Immunoglobulin M; *CD4*=Cluster of differentiation 4; *MHC-II* =Major histocompatibility complex class II. CG=control group; HIB=control diet + 1.0×10^8 CFU/g heat-

inactivated *B. pumilus* SE5; PG=control diet +21.30 mg/kg PG; LTA=control diet +6.70 mg/kg LTA; PL1=control diet +10.65 mg/kg PG +3.35 mg/kg LTA; PL2=control diet +21.30 mg/kg PG +6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

about their combined effects in aquaculture. The present study evaluated the effects of the mixture of PG and LTA derived from probiotic *B. pumilus* SE5 on the growth performance, feed utilization, immune function, and intestinal health in grouper *E. coioides*. Results indicated that PG and LTA alone or in combination significantly improved the SGR, FCR, and survival rate. In accordance with the present results, our previous study has shown that PG and LTA improved the growth performance and digestive enzyme activities and intestinal function in *E. coioides* [18]. Therefore, the improved growth performance probably attributed to better nutrient absorption caused by improved digestive enzyme activity and intestinal development [26–30]. In

fact, the amylase and lipase activities in group PL2 were superior to that in groups PG and LTA, and the protease activity in group PL1 were superior to that in group PG. On the other hand, the microvilli length, microvilli width, muscle layer thickness, and goblet cells in group PL1 was superior to those in groups PG and LTA. Furthermore, the highest expression of tight junction (TJ) proteins Claudin3 and Occludin was observed in group PL1, facilitating the establishment of tight junctions among intestinal epithelial cells and ensuring the stability of the physical barrier [31]. Taken together, the *B. pumilus* SE5-derived PG and LTA demonstrated synergistic efficacy in enhancing the intestinal structural integrity and digestive enzyme activity, which may

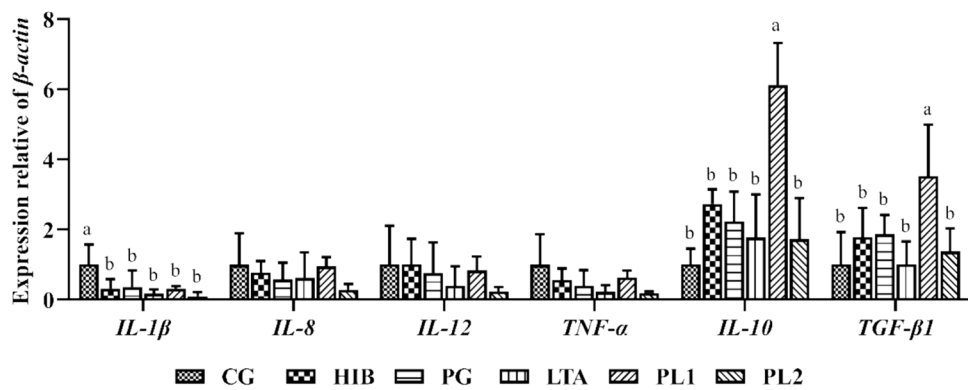


Fig. 10 The expression of pro-inflammatory cytokines (*IL-1 β* , *IL-8*, *IL-12* and *TNF- α*) and anti-inflammatory cytokines (*IL-10* and *TGF- β 1*) in the intestine of *E. coioides*. *IL-1 β* =interleukin-1 β ; *TNF- α* =tumor necrosis factor- α ; *IL-8*=interleukin-8; *IL-12*=interleukin-12; *IL-10*=interleukin-10; *TGF- β 1*=transforming growth factor β 1. CG=control group; HIB=control diet + 1.0×10^8 CFU/g

heat-inactivated *B. pumilus* SE5; PG=control diet +21.30 mg/kg PG; LTA=control diet +6.70 mg/kg LTA; PL1=control diet +10.65 mg/kg PG +3.35 mg/kg LTA; PL2=control diet +21.30 mg/kg PG +6.70 mg/kg LTA. Bars with the same superscripts are not significantly different ($P > 0.05$)

contribute to the improved growth performance and feed utilization in the grouper.

Intestinal microbiota plays a crucial role in regulating the immune system of fish, thereby boosting its resilience against pathogenic microorganisms. Concurrently, the host's immune system actively surveils the dynamics of the microbial community to maintain the intestinal microbiota homeostasis [32]. Probiotic and postbiotics supplementation has been suggested as an effective approach to shape the intestinal microbial composition, thereby promoting the growth and overall health of fish [33]. Our previous study demonstrated that *B. pumilus* SE5-derived PG and LTA could effectively modulate the overall structure of intestinal microbiota of the grouper [18]. Similarly, the *Lactobacillus plantarum*-derived LTA has been identified to be effective in preventing diseases associated with pathogenic *Staphylococcus aureus* by inhibiting the expression of the *ica*-operon and thereby inhibiting the production of poly-N-acetylglucosamine, which is required for biofilm development in *S. aureus* [34]. In the present study, the mixture groups (PL1 and PL2) increased the relative abundance of common beneficial Firmicutes while pathogenic Proteobacteria were decreased [35, 36]. At genus level, the mixture increased the relative abundance of *Lactobacillus* and *Lactococcus* (both belonging to Firmicutes) while decreasing the deleterious *Bacteroides* and *Staphylococcus*. Therefore, *B. pumilus* SE5-derived PG and LTA mixtures presented more effective impact on the improvement of gut microbial homeostasis, but the mechanisms are largely not clear, which deserve further study.

The remodeling intestinal microbiota by *B. pumilus* SE5-derived PG and LTA prompted us to inquire about any potential alterations in the functioning of the immune system. In fact, multiple studies have substantiated the impact of postbiotic stimulation of the intestinal immunity [37], which relies on the presence of T-lymphocytes in the epithelium and lamina propria, alongside B-lymphocytes, plasma cells, dendritic cells, macrophages, and granulocytes [38]. There are intricate intercellular interactions involving the initiation of the response by T lymphocytes, the effects of which are amplified by the binding of MHC (MHC class I and II) to T cell co-receptors (CD4 and CD8), which are considered to protect the fish from pathogen invasion [39]. In the present study, a slightly elevated expression of MHCII and CD4 was found with administration of PG and LTA alone or in combination. IgM is a type of immunoglobulin primarily secreted by B lymphocytes [40]; its upregulation expression was observed in the grouper fed with diets supplemented with *B. pumilus* SE5-derived PG or LTA diets in our previous study [18]. In this study, a higher relative expression of IgM was observed in group PL1 compared to that in groups PG and LTA. On the other hand, antimicrobial peptides (AMPs) represent a category of small molecular peptide compounds endowed with dual functionality: they can directly eliminate

pathogenic microorganisms, regulate immune responses, and facilitate mucosal repair [41]. The upregulated AMPs, such as epinecidin-1, hepcidin-1, and β -defensin, were observed in fish fed diets supplemented with PG and LTA alone, and the most pronounced effect was observed in group PL1. Therefore, the combination of PG and LTA could potentially activate the expression of antimicrobial effectors and IgM and thus maintain the general health of the grouper, which in turn may benefit their growth performance.

Previous research has demonstrated that pattern-recognition receptors (PPRs), such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), are capable of recognizing microbial-associated molecular patterns (MAMPs) released by microbes/probiotics [42], and these receptors subsequently trigger signaling cascades that intricately regulate the production of antimicrobial peptides (AMPs) and other effectors [43]. Peptidoglycan of potentially pathogenic bacteria is recognized by NOD2, which in consequence activates MAPK signaling thus inducing the secretion of antimicrobial peptides in human Paneth cells [44]. Our previous study observed that *B. pumilus* SE5-originated PG and LTA could enhance TLR2 and NOD2 expressions in *E. coioides* [16]. In this study, the mixture of PG and LTA is more effective in inducing the expression of NOD2 and TLR2 in the grouper. In addition, the expression of ERK1 was downregulated in groups LTA and PL2, which in line with a previous study indicated that *Lactobacillus paracasei*-derived LTA could inhibit ERK signaling pathways, thereby suppressing the release of cytokines [45]. Previous studies have found that *Lactobacillus plantarum* and *Staphylococcal*-derived lipopeptide LP01 could be recognized by TLR2 receptors and stimulate MAPK signaling pathway to regulate antimicrobial peptide synthesis, respectively [46, 47]. Therefore, we postulated that either PG and LTA alone or in combination can activate the expression of TLR2 and NOD2 and stimulate the P38MAPK pathway to promote the secretion of antimicrobial peptides thus shaping the intestinal microbiota and further maintaining intestinal health in grouper, and the mixture of PG and LTA showed better potential compared to PG or LTA alone.

The regulation of intestinal microbiota and reinforcement of immune function are crucial for the prevention and treatment of intestinal inflammation [48], which is closely related to the activation of the NF- κ B pathway [49]. The inhibition of NF- κ B kinase and the alleviation of *Shigella fowleri*-induced inflammation were demonstrated with *Lactobacillus plantarum*-derived LTA [50]. Our results showed that PG and LTA alone or in combination likely acts to inhibit the NF- κ B pathway, with downregulation of the TLR1, TLR3, TLR22, MyD88, TAK1, IKK α , IKK β , and NF- κ B genes, and the most pronounced suppression efficacy occurred in group PL2. Similarly, the *L. paracasei* D35-derived PG and

LTA could inhibit NF- κ B pathways thus alleviating inflammation [51, 52]. Pro-inflammatory cytokines such as IL-8 and TNF- α have also been described to be downregulated by *Lactobacillus plantarum*-derived LTA in human intestinal epithelial Caco-2 cells [53]. Furthermore, *Lactobacillus acidophilus*-derived LTA has been reported to downregulate IL-12 and TNF- α expressions in mice while upregulating IL-10 expression [54]. In the present study, the combination of PG and LTA could upregulate the expression of IL-10 and TGF- β 1 while inhibiting IL-1 β , and the highest expression of IL-10 and TGF- β 1 was observed in group PL1. Taken together, the administration of the PG and LTA mixture in diet could ameliorate inflammation by inhibiting the NF- κ B pathway and thus protecting the intestinal mucosal barrier and ultimately enhancing intestinal health of grouper.

In summary, compared to the individual addition of PG and LTA to feed, the mixture (10.65 mg/kg PG and 3.35 mg/kg LTA) demonstrates better efficacy in enhancing intestinal microbiota homeostasis and immune function, preventing enteritis and improving growth performance and feed utilization in *E. coioides*. These results provide valuable insights into the interactions between commensals and host fish and also lay the necessary foundation for the application of postbiotics in grouper aquaculture.

Author Contribution Jianming Xu: conceptualization, methodology, data curation, and original draft preparation; Guohe Cai: methodology and data curation; Jie Li: data curation and analysis; Hongling Yang: data curation and analysis; Jidan Ye: data analysis; Yunzhang Sun: supervision, conceptualization, methodology, validation, reviewing, and editing.

Funding This work was supported by the National Natural Science Foundation of China (32072990), Natural Science Foundation of Xiamen (3502Z202373031), Fujian Province agricultural guidance (key) project (2023N0012), Natural Science Foundation of Fujian Province (2021J05157), Xiamen Marine and Fisheries Development Fund (19CZP018HJ04), Science and Technology Major/Special Project of Fujian Province (2021NZ029022), and Educational Research Program for Young and Middle-aged Teachers of Fujian Provincial Department of Education (JAT200231).

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

References

- Liu X, Steele JC, Meng XZ (2017) Usage, residue, and human health risk of antibiotics in Chinese aquaculture: a review. *Environ Pollut* 223:161–169
- Zhao Y, Yang QE, Zhou X, Wang F, Muurinen J, Virta MP, Brandt KK, Zhu Y (2020) Antibiotic resistome in the livestock and aquaculture industries: status and solutions. *Crit Rev Environ Sci Technol* 51(19):2159–2196
- Limbu SM, Chen LQ, Zhang ML, Du ZY (2020) A global analysis on the systemic effects of antibiotics in cultured fish and their potential human health risk: a review. *Rev Aquac* 13(2):1015–1059
- Van Doan H, Hoseinifar SH, Ringø E, Ángeles Esteban M, Dadar M, Dawood MAO, Faggio C (2019) Host-associated probiotics: A key factor in sustainable aquaculture. *Rev Fish Sci Aquac* 28(1):16–42
- Hoseinifar SH, Sun YZ, Wang A, Zhou Z (2018) Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Front Microbiol* 9:2429
- Wang A, Ran C, Wang Y, Zhang Z, Ding Q, Yang Y, Olsen RE, Ringo E, Bindelle J, Zhou Z (2019) Use of probiotics in aquaculture of China—a review of the past decade. *Fish Shellfish Immunol* 86:734–755
- Merenstein D, Pot B, Leyer G, Ouwehand AC, Preidis GA, Elkins CA, Hill C, Lewis ZT, Shane AL, Zmora N (2023) Emerging issues in probiotic safety: 2023 perspectives. *Gut microbes* 15(1):2185034
- Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, Sanders ME, Shamir R, Swann JR, Szajewska H, Vinderola G (2021) The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat Rev Gastroenterol Hepatol* 18(9):649–667
- Sudhakaran G, Guru A, Haridevamuthu B, Murugan R, Arshad A, Arockiaraj J (2022) Molecular properties of postbiotics and their role in controlling aquaculture diseases. *Aquac Res* 53(9):3257–3273
- Casadei E, Bird S, Wadsworth S, Vecino JLG, Secombes CJ (2015) The longevity of the antimicrobial response in rainbow trout (*Oncorhynchus mykiss*) fed a peptidoglycan (PG) supplemented diet. *Fish Shellfish Immunol* 44(1):316–320
- Zhou J, Song X-L, Huang J, Wang X-H (2006) Effects of dietary supplementation of A3 α -peptidoglycan on innate immune responses and defense activity of Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 251(2–4):172–181
- Zhang C, Chen G, Wang CC, Song X, Wang Y, Xu Z (2014) Effects of dietary supplementation of A3 α -peptidoglycan on the growth, immune response and defence of sea cucumber *Apostichopus japonicus*. *Aquac Nutr* 20(2):219–228
- Gao Q, Gao Q, Min M, Zhang C, Peng S, Shi Z (2016) Ability of *Lactobacillus plantarum* lipoteichoic acid to inhibit *Vibrio anguillarum*-induced inflammation and apoptosis in silvery pomfret (*Pampus argenteus*) intestinal epithelial cells. *Fish Shellfish Immunol* 54:573–579
- Lazado CC, Caipang CMA (2014) Mucosal immunity and probiotics in fish. *Fish Shellfish Immunol* 39(1):78–79
- Sun Y, Yang H, Ma R, Lin W (2010) 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(5):803–809
- Yang HL, Hu X, Ye JD, Seerengaraj V, Yang W, Ai CX, Sun YZ (2021) Cell wall components of *Bacillus pumilus* SE5 improved the growth, digestive and immunity of grouper (*Epinephelus coioides*). *Curr Chin Sci* 1(2):231–239
- Sun Y, Yang H, Ling Z, Chang J, Ye J (2009) Gut microbiota of fast and slow growing grouper *Epinephelus coioides*. *African journal of microbiology research* 3(11):713–720
- Yang H, Sun Y, Hu X, Ye J, Lu K, Hu L, Zhang J (2019) *Bacillus pumilus* SE5 originated PG and LTA tuned the intestinal TLRs/MyD88 signaling and microbiota in grouper (*Epinephelus coioides*). *Fish Shellfish Immunol* 88:266–271

19. Morath S (2001) Structure–function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. *J Exp Med* 193(3):393–398
20. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226(1):497–509
21. Yang HL, Xia HQ, Ye YD, Zou WC, Sun YZ (2014) Probiotic *Bacillus pumilus* SE5 shapes the intestinal microbiota and mucosal immunity in grouper *Epinephelus coioides*. *Dis Aquat Organ* 111(2):119–127
22. Sun Y, Yang H, Ma R, Lin W (2010) 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(5):803–809
23. Liu Z, Yang H, Hu L, Yang W, Ai C, Sun Y (2021) Dose-dependent effects of histamine on growth, immunity and intestinal health in juvenile grouper (*Epinephelus coioides*). *Front Mar Sci* 8:685720
24. Sun YZ, Yang HL, Ma RL, Song K, Lin WY (2011) Molecular analysis of autochthonous microbiota along the digestive tract of juvenile grouper *Epinephelus coioides* following probiotic *Bacillus pumilus* administration. *J Appl Microbiol* 110(4):1093–103
25. Sun Y, Xia H, Yang H, Wang Y, Zou W (2014) TLR2 signaling may play a key role in the probiotic modulation of intestinal microbiota in grouper *Epinephelus coioides*. *Aquaculture* 430:50–56
26. Sagada G, Gray N, Wang L, Xu B, Zheng L, Zhong Z, Ullah S, Tegomo AF, Shao Q (2021) Effect of dietary inactivated *Lactobacillus plantarum* on growth performance, antioxidative capacity, and intestinal integrity of black sea bream (*Acanthopagrus schlegelii*) fingerlings. *Aquaculture* 535:736370
27. Arias Padro MD, Caboni E, Salazar Morin KA, Meraz Mercado MA, Olalde-Portugal V (2021) Effect of *Bacillus subtilis* on antioxidant enzyme activities in tomato grafting. *Peer J* 9:e10984
28. Won S, Hamidoghli A, Choi W, Park Y, Jang WJ, Kong I, Bai SC (2020) Effects of *Bacillus subtilis* WB60 and *Lactococcus lactis* on growth, immune responses, histology and gene expression in Nile tilapia *Oreochromis niloticus*. *Microorganisms* 8(1):1–16.
29. Xia Yun Lu, Maixin Chen Gang, Jianmeng Cao, Fengying Gao (2018) Effects of dietary *Lactobacillus rhamnosus* JCM1136 and *Lactococcus lactis* subsp lactis JCM5805 on the growth, intestinal microbiota, morphology, immune response and disease resistance of juvenile Nile tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol* 76:368–379
30. Pluske JR, Thompson MJ, Atwood CS, Bird PH, Williams IH, Hartmann PE (2005) Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *Br J Nutr* 76(3):409–422
31. Ohland CL, Macnaughton WK (2010) Probiotic bacteria and intestinal epithelial barrier function. *Am J of Physiol Gastrointest Liver Physiol* 298(6):807–819
32. Dawood MA (2021) Nutritional immunity of fish intestines: important insights for sustainable aquaculture. *Rev Aquac* 13(1):642–663
33. Ringø E, Zhou Z, Vecino JG, Wadsworth S, Romero J, Krogdahl Å, Olsen RE, Dimitroglou A, Foey A, Davies S (2016) Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquac Nutr* 22(2):219–282
34. Ahn KB, Baik JE, Yun CH, Han SH (2018) Lipoteichoic acid inhibits *Staphylococcus aureus* biofilm formation. *Front Microbiol* 9:327
35. Parma L, Yúfera M, Navarro-Guillén C, Moyano FJ, Soverini M, D'Amico F, Candela M, Fontanillas R, Gatta PP, Bonaldo A (2019) Bonaldo, Effects of calcium carbonate inclusion in low fishmeal diets on growth, gastrointestinal pH, digestive enzyme activity and gut bacterial community of European sea bass (*Dicentrarchus labrax L.*) juveniles. *Aquaculture* 510:283–292
36. Yang H, Bian Y, Huang L, Lan Q, Ma L, Li X, Leng X (2022) Effects of replacing fish meal with fermented soybean meal on the growth performance, intestinal microbiota, morphology and disease resistance of largemouth bass (*Micropterus salmoides*). *Aquaculture Reports* 22:100954
37. Mehta JP, Ayakar S, Singhal RS (2023) The potential of paraprobiotics and postbiotics to modulate the immune system: a review. *Microbiol Res* 275:127449
38. Nayak SK (2010) Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol* 29(1):2–14
39. Dee CT, Nagaraju RT, Athanasiadis EI, Gray C, Fernandez del Ama L, Johnston SA, Secombes CJ, Cvejic A, Hurlstone AF (2016) CD4-transgenic zebrafish reveal tissue-resident Th2- and regulatory T cell-like populations and diverse mononuclear phagocytes. *J Immunol* 197(9):3520–3530
40. Bilal S, Lie KK, Karlsen OA, Hordvik I (2016) Characterization of IgM in Norwegian cleaner fish (*lumpfish and wrasses*). *Fish Shellfish Immunol* 59:9–17
41. Muniz LR, Knosp C, Yeretsian G (2012) Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front Immunol* 3:32484
42. Alghsham R, Rasheed Z, Shariq A, Alkhamiss AS, Alhumaydhi FA, Aljohani AS, Althwab SA, Alshomar A, Alhomaidean HT, Hamad EM (2022) Recognition of pathogens and their inflammatory signaling events. *Open Access Maced J Med Sci (OAMJMS)* 10:462–467
43. Liu Y, Shepherd EG, Nelin LD (2007) MAPK phosphatases—regulating the immune response. *Nat Rev Immunol* 7(3):202–212
44. Lala SG, Ogura Y, Hor SK, Abeya M, Keshav S (2003) Crohn's disease and the NOD2 gene A role for Paneth cells. *Annual Meeting of the British-Society-of-Gastroenterology* 125(1):47–57
45. Zhang L, Liu J, Kong S, Chen N, Hung W-L, Zhao W, Zeng Z, Zhang J, Yang Z (2023) Lipoteichoic acid obtained from *Lactobacillus paracasei* via low-temperature pasteurization alleviates the macrophage inflammatory response by downregulating the NF-κB signaling pathway. *J Funct Foods* 107:105673
46. Wang J, Zhang W, Wang S, Liu H, Zhang D, Wang Y, Ji H (2019) Swine-derived probiotic *Lactobacillus plantarum* modulates porcine intestinal endogenous host defense peptide synthesis through TLR2/MAPK/AP-1 signaling pathway. *Front Immunol* 10:2691
47. Li D, Lei H, Li Z, Li H, Wang Y, Lai Y (2013) A novel lipopeptide from skin commensal activates TLR2/CD36-p38 MAPK signaling to increase antibacterial defense against bacterial infection. *PLOS ONE* 8(3):e58288
48. Belkaid Y, Hand TW (2014) Role of the microbiota in immunity and inflammation. *Cell* 157(1):121–141
49. Hayden MS, Ghosh S (2011) NF-κB in immunobiology. *Cell Res* 21(2):223–244
50. Kim HG, Lee SY, Kim NR, Lee HY, Ko MY, Jung BJ, Kim CM, Lee JM, Park JH, Han SH, Chung DK (2010) *Lactobacillus plantarum* lipoteichoic acid down-regulated *Shigella flexneri* peptidoglycan-induced inflammation. *Mol Immunol* 48(4):382–391
51. Wang S, Ahmadi S, Nagpal R, Jain S, Mishra SP, Kavanagh K, Zhu X, Wang Z, McClain DA, Kritchevsky SB, Kitzman DW, Yadav H (2020) Lipoteichoic acid from the cell wall of a heat killed *Lactobacillus paracasei* D3–5 ameliorates aging-related leaky gut, inflammation and improves physical and cognitive functions: From *C. elegans* to mice. *Geroscience* 42(1):333–352
52. Wang J, Zhang W, Wang S, Liu H, Zhang D, Wang Y, Ji H (2019) Swine-derived probiotic *Lactobacillus plantarum* modulates porcine intestinal endogenous host defense peptide synthesis through TLR2/MAPK/AP-1 signaling pathway. *Front Immunol* 10:2691
53. Su YN, Kang SS, Yun CH, Han SH (2014) Lipoteichoic acid from *Lactobacillus plantarum* inhibits Pam2CSK4-induced IL-8

production in human intestinal epithelial cells. *Mol Immunol* 64(1):183-189.

54. Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Pakanati KC, Ansari MJ, O'Flaherty S, Barrett T, Klaenhammer TR (2011) Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. *Proc Natl Acad Sci USA* 108(Suppl 1):4623–30

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