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Xylanase enhances gut microbiota-derived butyrate to exert immune-protective effects in a histone deacetylase-dependent manner

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Abstract

Background Commensal bacteria in the intestine release enzymes to degrade and ferment dietary components, producing beneficial metabolites. However, the regulatory effects of microbial-derived enzymes on the intestinal microbiota composition and the influence on host health remain elusive. Xylanase can degrade xylan into oligosaccharides, showing wide application in feed industry.

Results To validate the immune-protective effects of xylanase, Nile tilapia was used as the model and fed with xylanase. The results showed that dietary xylanase improved the survival rate of Nile tilapia when they were challenged with *Aeromonas hydrophila*. The transcriptome analysis showed significant enrichment of genes related to interleukin-17d (*il-17d*) signaling pathway in the xylanase treatment group. High-throughput sequencing revealed that dietary xylanase altered the composition of the intestinal microbiota and directly promoted the proliferation of *Allobaculum stercoricanis* which could produce butyrate in vitro. Consequently, dietary xylanase supplementation increased the butyrate level in fish gut. Further experiment verified that butyrate supplementation enhanced the expression of *il-17d* and regenerating islet-derived 3 gamma (*reg3g*) in the gut. The knockdown experiment of *il-17d* confirmed that *il-17d* is necessary for butyrate to protect Nile tilapia from pathogen resistance. Flow cytometry analysis indicated that butyrate increased the abundance of IL-17D$^+$ intestinal epithelial cells in fish. Mechanistically, butyrate functions as an HDAC3 inhibitor, enhancing *il-17d* expression and playing a crucial role in pathogen resistance.

Conclusion Dietary xylanase significantly altered the composition of intestinal microbiota and increased the content of butyrate in the intestine. Butyrate activated the transcription of *il-17d* in intestinal epithelial cells by inhibiting histone deacetylase 3, thereby protecting the Nile tilapia from pathogen infection. This study elucidated how microbial-derived xylanase regulates host immune function, providing a theoretical basis for the development and application of functional enzymes.

Keywords Pathogen resistance, Xylanase, Intestinal microbiota, Short-chain fatty acids, Histone deacetylation

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Introduction

Gut microbiota is a diverse and complex ecosystem that plays a crucial role in maintaining host physiological processes and immune system function [1]. It has been documented that commensal bacteria can ferment diet ingredients to produce beneficial metabolites or influence the ecological network of microbiota to affect the host health [2–4]. Therefore, selective manipulation of the gut microbiota is an effective approach to enhance immune status and overall host health [5]. Current strategies for microbiota manipulation include the use of prebiotics, probiotics, or fecal microbiota transplantation [6]. Enzymes, the main products of some specific microbiota, could also regulate intestinal microbial composition and immunity status of animals [7–9].

Gut microbes released enzymes to facilitate cross-feeding, which plays a pivotal role in shaping the composition of community and the metabolic characteristics [10, 11]. Microbiota depend on fellow members within the community to obtain substrates, such as carbohydrate, electron donors, and amino acids, for their growth or production of metabolites [11]. For example, *Bacteroides ovatus* or *Lactobacillus paracasei* secrete enzymes to metabolize polysaccharides like inulin and xylan into monosaccharides, which are subsequently fermented by *Faecalibacterium prausnitzii* and *Bacteroides vulgatus* to produce beneficial metabolites such as short-chain fatty acids [12–14]. In this model, microbial-derived enzymes impact the microbiota composition, but interactions among enzymes, microbial cross-feeding, and host immune status remain elusive.

Microbial metabolic products could interact with intestinal epithelial cells and thus improve host immunity and pathogen resistance [15]. Intestinal epithelial cells (IECs) serve as both chemical and physical barriers, playing a crucial role in maintaining intestinal homeostasis [16]. In mammals, IECs are equipped with pattern-recognition receptors (PRRs), empowering them to serve as dynamic sensors of the microbial environment. They actively directed mucosal immune cell responses [16]. Furthermore, a specific type of intestinal epithelial cell called Paneth cells secretes antimicrobial proteins (AMPs), which act as innate immune effectors to defend against pathogens [17].

Fish, encompassing nearly half of all vertebrate diversity, serve as a vital group for comprehending the evolution and ecology of host-microbiota interactions [18]. In fish, the anterior, middle, and posterior intestine exhibit unique functionalities akin to those of the small and large intestines in mammals [19]. However, fish intestines lack crypts, Paneth cells, and submucosal glands [20], suggesting that in lower vertebrates, functional specialization precedes morphological regionalization. Therefore, studying the function of fish gut microbiota and intestinal epithelial cells in pathogen resistance can fill the gap in our understanding of the host-microbe interaction in vertebrate.

Xylanase can be secreted by many commensal bacteria, including *Bacillus*, *Streptomyces*, and *Ruminococcus* [21], and is one of the commonly used enzymes in feeding industry [22, 23]. Xylanase hydrolyzes xylan into oligosaccharides, resulting in alterations in intestinal microbiota composition. However, how xylanase changes the ecological networks of intestinal microbiota and improves host health remains unknown. In this study, we uncovered that xylanase enriched the abundance of butyrate-producing intestinal microbe and increased the content of butyrate in the intestine. Butyrate acts as the histone deacetylase inhibitor to activate the transcription of *il-17d* in intestinal epithelial cells, thereby protecting Nile tilapia from pathogen infection.

Methods

Experimental procedures, experimental diets, and tissue embedding

Xylanase addition experiment

One-hundred eighty juvenile healthy Nile tilapia (3.89 ± 0.01 g) were divided into 2 groups, and each group contained 3 tanks (30 fish/tank). The fish were fed with soybean meal-based diet (SM), or soybean meal-based diet supplemented with 3000 U/kg xylanase (SMC) respectively, by hand-feed twice daily for 8 weeks. The xylanase used in this study was derived from *Caldicellulosiruptor bescii*. The xylanase was recombinantly expressed in the *Pichia pastoris* GS115-pPIC9γ [24].

The composition of two experimental diets was listed in Table S1. The experimental diets were stored at − 20 °C until use. At the end of trial, 12 fish were randomly selected from SM and SMC groups. The blood was drawn from the caudal vein and centrifuged at 3000 r/min for 10 min at 4 °C. The separated serum was stored at − 80 °C. The entire intestinal content was separated from the gut lumen by tweezers and stored at − 80 °C for sequencing. The intestinal tissue was used for transcriptome analysis (Figure S1).

Sodium butyrate addition experiment

In the supplementation experiment of sodium butyrate (156–54-7, Merck & Co., Inc., USA), 180 Nile tilapia (3.03 ± 0.02 g) were divided into 2 groups, each group with 3 tanks (30 fish/tank). Fish were fed with soybean meal-based diet (SM) or soybean meal-based diet supplemented with 40 mmol/kg sodium butyrate (SB) twice daily for 8 consecutive weeks. The feed formulations were listed in Supplementary Table S2. At the end of experiment, the fish were anesthetized with MS-222. Nine fish

were randomly selected from each group. The blood was collected and stored at − 80 °C. Intestinal tissue was collected for further analysis (Figure S2).

Challenge experiment

Aeromonas hydrophila (CGMCC 1.2017) was cultured in Luria Broth (LB) at 28 °C for 16 h. The fish were intraperitoneally injected with *A. hydrophila* at 1×10^6 CFU/g as described in our previous study [25]. The diet fed to Nile tilapia during the challenge stage was the same as that used during the cultivation stage. The mortality of each group was recorded until the fish reached a stable condition.

Transcriptome analysis

RNA was isolated from the intestinal tissue of Nile tilapia using TRIzol reagent (Invitrogen, USA). The library construction required a total RNA amount of 1 μg, a concentration of ≥ 30 ng/μL, and an OD260/280 ratio between 1.8 and 2.2. The library construction and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). The library was quantified using Qubit 4.0 and sequenced on the NovaSeq X Plus platform. The clean data for each sample exceeded 6.07 Gb and were aligned to the Oreochromis niloticus reference genome (GCF_001858045.2) and assembled. Sequence alignment analysis was performed using HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml). Gene expression for each sample was calculated using the transcripts per million reads (TPM) method to identify differentially expressed genes between the SM and SMC groups. Differential gene expression analysis was conducted using DESeq2, with screening thresholds set at a *P*-value < 0.05 and log2 fold change ≥ 1.5. KEGG pathway enrichment analysis was performed using an R script, applying Fisher's exact test with multiple testing correction via the Benjamini–Hochberg method. A corrected *P*-value of less than 0.05 indicated significant enrichment of the KEGG pathway function. Default parameters were used for any unlisted settings.

Quantitative real-time PCR (qPCR)

TRIzol Reagent was used to extract RNA from the intestinal tissue of Nile tilapia. The concentration of RNA and the ratio of A260/280 were measured using a NanoDrop. The quantity and integrity of RNA were analyzed via agarose gel electrophoresis. Reverse transcription was performed with HiScript IV RT SuperMix (R423, Vazyme Biotech Co., Ltd., China) according to the manufacturer's instructions. The reaction condition of qPCR contained 1-μL cDNA, 1 μL 0.2-μM primers, 5-μL qPCR SYBR Green Master Mix, and 3-μL RNase- and DNase-free water. Elongation factor-1 alpha (*ef1α*) was used as the reference gene to calculate target gene relative expression by $2^{-\Delta\Delta CT}$ method. The sequences of primers were listed in Table S3.

Enzyme-linked immunosorbent assay

The levels of IL-17D (HB1082-QT) and Reg3γ (HB1120-QT) in serum were detected by ELISA using antibodies specific for Nile tilapia according to the manufacturer's protocol (Shanghai Hengyuan Biotechnology Co., Ltd., Shanghai, China).

Establishment of il-17d knockdown Nile tilapia

The intestinAL cDNA was used as the template to synthesize dsDNA *il-17d* with specific amplification primers (*il-17d*-iF-TCAGTCGGGGTTTTACGCAC and *il-17d*-iR-GTCCTGACGGTCTGCTTTCT). dsRNA *il-17d* was synthesized using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Shanghai, China) according to the manufacturer's instructions. The knockdown efficiency of dsRNA was approximately 50% (Figure S3).

To investigate the necessity of il17d for the immune function of xylanase and butyrate, we conducted a new rearing experiment following the same method as described in the "Experimental procedures, experimental diets, and tissue embedding" section. Thirty fish (10 fish per tank) from each group were injected with normal saline solution (0.86%) or 5 μg/g body weight dsRNA of *il-17d*, respectively. Among them, 30 fish were challenged with *A. hydrophila*, and another 6 fish were sampled for gene expression detection at 24-h post-injection.

High-throughput sequencing

Total DNA was extracted from intestinal contents in SM and SMC groups using QIAamp DNA Stool Mini Kit 51,604 (QIAGEN, Hilden, Germany). The DNA concentration was detected with NanoDrop 2000 (Thermo Scientific, Waltham, USA), and the purity of DNA was checked via 1% agarose electrophoresis. The 16S rRNA gene of six fish from SM and SMC groups were amplified with the primers targeting V3–V4 regions (338F 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′) and analyzed on the Illumina MiSeq platform (Personal Biotechnology Co., Ltd., Shanghai, China). The 16S rRNA of intestinal microbiota of 12 fish from each treatment was sequenced on the PacBio Sequel platform by using primers for the full length of 16S rRNA gene (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′).

The sequences were then quality filtered, denoised, merged, and chimera removed by the QIIME2 [26]. Alpha-diversity metrics were calculated by Chao1, Faith,

Goods, Shannon, Pielou, and Simpson. Phylogenetic tree plot was performed using the classify-sklearn algorithm (https://github.com/QIIME2/q2-feature-classifier) in QIIME2 (2019.4) software with default parameters. The phylogenetic tree was plotted using the Wilcoxon rank-sum test and R language. Differences in species abundance between the SM and SMC groups were analyzed using Student's *t*-test, with significance determined by a Benjamini–Hochberg corrected *P*-value of < 0.05. Principal component analysis (PCA) was performed by R software. Correlation network analysis was constructed using igraph, based on the top 50 nodes by average abundance, and visualized using the ggraph (version 1.2.7) package.

Bacterial culture and bacteria-bacteria interactions

The *Lactobacillus plantarum* and *Plesiomonas shigelloides* strains were isolated from the intestinal content of Nile tilapia (Supplementary material 1.1). The *Akkermansia muciniphila* (DSM 22959) and *Allobaculum stercoricanis* (DSM 13633) were purchased from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). PYG medium was used for culturing *L. plantarum*, *P. shigelloides*, and *A. stercoricanis*, while *A. muciniphila* was cultured in BHI medium containing 0.1% mucin. All the bacteria were cultured in 25-mL anaerobic tubes for 24 h. The culture supernatants were collected by centrifuging at 4000 rpm for 10 min. To explore the effects of xylanase on the proliferation of bacteria, soybean meal was used to replace the glucose in the PYG and BHI medium, and the OD_{600} of four bacteria was measured with or without xylanase supplementation. To explore the interactions between bacteria, the 10-mL culture supernatant of *L. plantarum*, *P. shigelloides*, or *A. muciniphila* was used to culture *A. stercoricanis* in PYG medium. The culture supernatant of *A. stercoricanis* was added to PYG medium for culturing *L. plantarum* and *P. shigelloides* and BHI medium for culturing *A. muciniphila*. After 24 h culturing, the OD_{600} of bacteria was measured.

Short-chain fatty acid determination

The gut contents from every two fish were pooled, and 0.2 g of the pooled gut contents was used for SCFA determination. The intestinal content and 1-ml bacteria culture supernatant were pooled and acidified with 50% sulfuric acid and then extracted with 300-μL ether. Gas chromatography (Nexis GC-2010 Plus, Shimadzu, Kyoto, Japan) was used to analyze the levels of SCFAs [27].

The effects of A. stercoricanis on the gene expression of il-17d

A. stercoricanis were cultured in PYG medium for 20 h. The culture supernatant was collected by centrifuging at 4000 rpm for 10 min and then freeze-dried for 24 h. The bacteria were collected and heat inactivated at 85 °C for 1 h. A total of 1-ml ($2\times10^{\wedge8}$ CFU) bacterial culture supernatant and heat-inactivated bacterial cells were used to treat primary intestinal cells for 12 h for gene expression detection.

Intestinal epithelial cell isolation and culture

The whole intestinal tissue was incubated with 5% FBS, 2% penicillin and streptomycin, and DMEM medium (10,564,011, Thermo Fisher Scientific Inc., USA) containing 1 mg/ml collagenase IV and 0.5% DNase for 90 min. The intestinal cells were grinded and filtrated through a nylon mesh in DMEM medium. Lymphocytes were collected from between 34 and 51% Percoll layers (17,089,109, GE HealthCare, USA), and the remaining cells were filtered through a 70-μm cell strainer to obtain intestinal epithelial cells. A total of 1-mM sodium butyrate, 0.1-μM FFAR2 inhibitor (GC31614, MedChemExpress, USA), 3-mM FFAR3 inhibitor (52,017, Merck & Co., Inc., USA), 1-μM trichostatin A (TSA, GC15526, GlpBio Technology Inc., USA), 10-μM nicotinamide (NAM, GN10347, GlpBio Technology Inc., USA), 1-μM ITSA (HY-100508, GlpBio Technology Inc., USA), and 2-μM RGFP966 (GC14285, GlpBio Technology Inc., USA) were added to intestinal epithelial cells for 12 h to detect the gene level of *il-17d*.

Flow cytometry analysis

Intestinal cells, lymphocytes, and intestinal epithelial cells were stained with LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (L34969, Thermo Fisher Scientific Inc., USA) for 30 min. After washing twice with Perm/Wash solution (51-2091KZ, Becton, Dickinson and Company, USA), the cells were fixed with BD Cytofix/Cytoperm buffer (51-2090KZ, Becton, Dickinson and Company, USA) for 30 min at 4 °C and washed twice. Subsequently, cells were incubated with IL-17D (MAB2274, R&D, USA) for 30 min and stained with IgG H&L (Alexa Fluor® 647, ab150115, Abcam plc, China) for 30 min at 4 °C. The samples were detected by a BD FACSCalibur flow cytometer, and the data were further processed with FlowJo software.

Western blots

Intestine samples were lysed with radio immunoprecipitation assay (RIPA) reagent containing the protease/phosphatase inhibitor and centrifuged at 12,000 rpm for 10 min to obtain lysates. Equal amount of protein sample was separated by 10% SDS-PAGE (New Cell & Molecular Biotech, China) and transferred to nitrocellulose membranes. After blocked with QuickBlock™ Western (P0252, Beyotime Biotechnology, China) for 20 min, the

membranes were incubated with the primary antibodies pan-acetylation (66,289–1-Ig, Proteintech, Rosemont, USA), HDAC3 (10,255–1-AP, Proteintech, Rosemont, USA), and α-tubulin (M1501-180, HUABIO, Hangzhou, China) overnight at 4 °C. Subsequently, membranes were incubated with anti-rabbit IgG (LI-COR Biotechnology, Nebraska, USA) or anti-mouse IgG (Li-Cor Biotechnology, Nebraska, USA) for 1 h and finally visualized using Odyssey CLx Imager (LI-COR, Inc.).

Luciferase activity assay

Il-17d promoter (Supplementary material 1.2) was constructed into pGL4.10-luc plasmids by gene synthesis. 1×10^5/mL 293 T cells were co-transfected with *Renilla* luciferase plasmids 20-ng pGMLR-TK and 600-ng pGL4.10- *il-17d* -luc plasmids for 24 h and then treated with sodium butyrate or RGFP966 for another 12 h. 293 T cells were lysed in luciferase reporter buffer, and the *Renilla* luciferase activity was used to normalize the luciferase activity. The relative luciferase activity was the value of luciferase activity/*Renilla* luciferase activity.

Statistical analysis

The data in the study were analyzed by Graph Prism 9.0 software and presented as mean ± SEM. Unpaired Student's test or one-way ANOVA was used for analyzing the difference between treatments. The log-rank test was used in the survival experiment. *P*-values < 0.05 (*) were considered statistically significant.

Results

Xylanase improve the pathogen resistance of Nile tilapia by activating IL-17D production in intestine

To determine whether xylanase has the protective effect against pathogen, Nile tilapia was challenged with *A. hydrophila* at the end of the trial (Fig. 1A). Compared with SM treatment, xylanase administration led to a higher survival rate, exhibiting protective the effect in Nile tilapia (Fig. 1B). To characterize the protective immune mechanism of xylanase, RNA-seq was conducted on the intestinal tissue of Nile tilapia. Substantial transcriptional changes related to immunity were observed between SM and SMC groups (Fig. 1C, Fig. S4A); of note, the *il-17d* signaling pathway was significantly changed in the comparison between the SM and SMC groups (Fig. 1D). The analysis also revealed that proinflammatory-associated genes, such as *hsp90b1* and *nfkbia*, were downregulated, whereas *il-17d* were upregulated in the SMC treatment (Fig. 1E). Consistent with the transcriptomic data, the expression of *il-17d* in the intestine and the content of IL-17D in serum were higher in the SMC group than those in the SM group (Fig. 1F). Then, we examined by qPCR the gene expression of *il-22* and *reg3g*, which are the downstream genes of *il-17d*. Compared with the SM group, the SMC group showed a marked increase in *il-22* and *reg3g* (Fig. S4B, Fig. 1G).

Given the importance of *il-17d* in gut homeostasis, dsRNA was used to knock down *il-17d* gene expression to assess the requirement of *il-17d* in the immunoprotective effect of xylanase treatment. Compared to the SMC group, the fish with *il-17d* gene silencing exhibited a lower survival rate when challenged with *A. hydrophila* (Fig. 1H). qPCR and ELISA were used to assess the expression of IL-17D and its downstream genes after *il-17d* gene silencing. The results showed that the gene level of *il-17d* and *reg3g* in the intestine, as well as the concentration of IL-17D and Reg3γ in serum, reduced in the dsRNA group (Fig. 1I, J, K). These data suggested that IL-17D played a critical immunoprotective role in fish when xylanase was added.

Xylanase altered intestinal microbiota composition

To investigate the change of intestinal composition, we used Illumina V3–V4 sequencing to analyze the intestinal bacterial composition. An average of 96,571 reads were obtained for each sample. V3–V4 Illumina data showed that the index of Chao1, Faith, Observed species, Shannon, and Pielou was higher in the SMC treatment than those in the SM treatment, while Simpson was similar in SM and SMC groups (Fig. 2A). The gut microbial composition was analyzed. The top 5 phyla in the SM group were Fusobacteria (79.23%), Proteobacteria (5.2%), Firmicutes (4.66%), Actinobacteria (4.15%), and Bacteroidetes (3.85%) (Fig. 2B). In the SMC group, Actinobacteria (34.2%), Proteobacteria (25.95%), Firmicutes (15.41%), Chloroflexi (5.82%), and Fusobacteria (4.86%) were the dominant phyla (Fig. 2B). The relative abundance of top 50 genus accounts for 69% of the total bacterial abundance. The relative abundance of the genus *Cetobacterium* was 78.78% in the SM group but significantly decreased to 4.8% in the SMC group (Fig. 2C). Xylanase administration increased the abundance of *Plesiomonas* and *Lactobacillus* with a tendency. Xylanase markedly increased the relative abundance of *Allobaculum* from 0.2% with the soybean meal-based diet to 1.5% (Fig. 2C). Principal coordinate analysis (PCoA) was performed to illustrate the clustering of microbial community in the different treatments. PCoA, which explains that the 45.7% (PCo1), 19.2% (PCo2) of the data in V3–V4 Illumina sequencing, clearly separates the microbial composition of the SM and SMC group (Fig. 2D). The correlation network analysis indicated that the genus of *Plesiomonas*, *Lactobacillus*, *Allobaculum*, and *Cetobacterium* belong to the same module_2 (Fig. 2 E, F).

To comprehensively analyze the effects of xylanase on the composition of the intestine, high-throughput

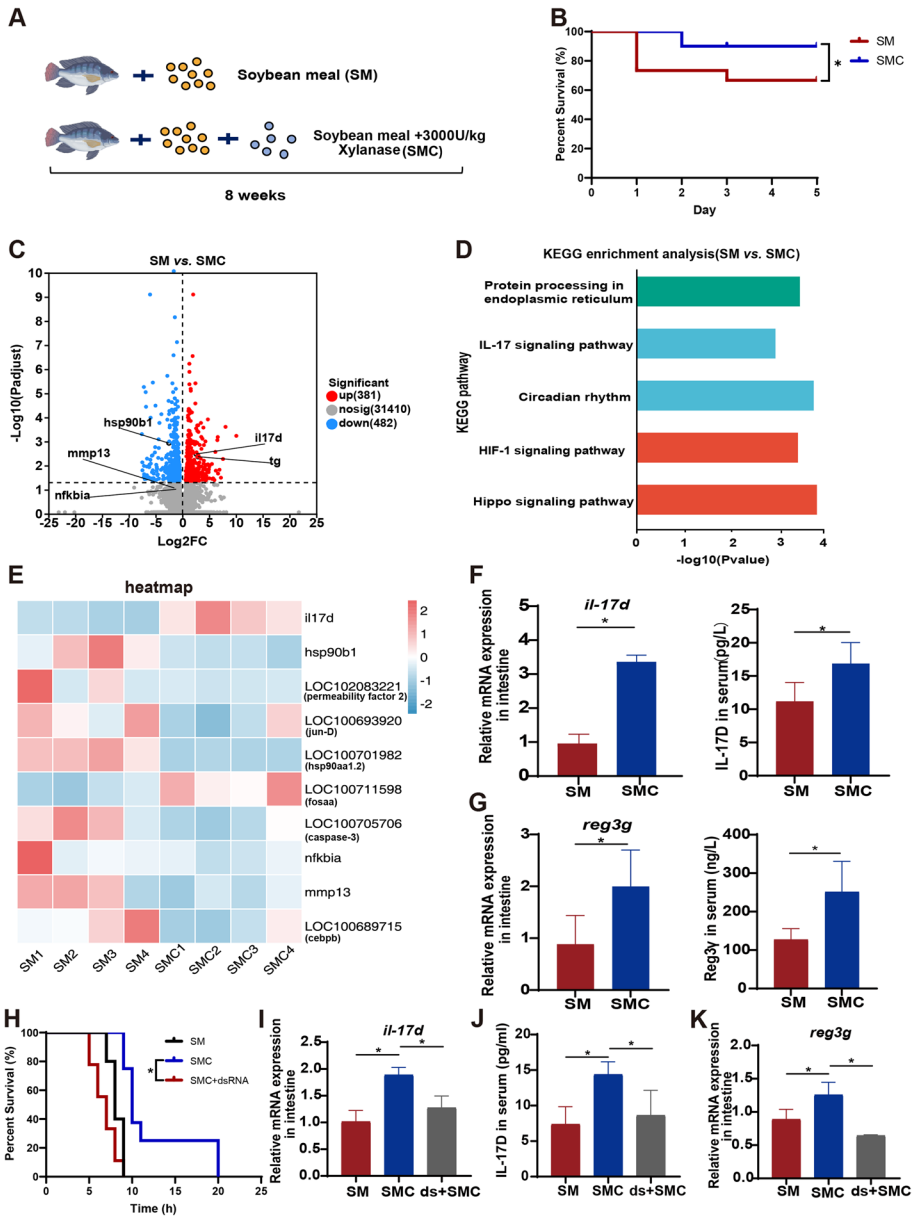

Fig. 1 Xylanase improved the pathogen resistance of Nile tilapia by regulating IL-17D-Reg3γ signaling pathway. **A** Experiment design. Nile tilapia was fed with soybean meal-based diet with or without 3000 U/kg xylanase for 8 weeks ($n=3$ tanks, 30 fish per tank). **B** Survival rate of Nile tilapia against *A. hydrophila* infection of SM and SMC group ($n=3$ tanks, 10 fish per tank). **C** The significantly upregulated and downregulated genes related to immunity between SM and SMC group ($n=4$). **D** The differently KEGG pathway between SM and SMC group ($n=4$). **E** The gene expression in *il-17* signaling pathway ($n=6$). **F** The gene expression of *il-17d* in the intestine and the concentration of IL-17D in serum ($n=6$). **G** The gene expression of *reg3g* in the intestine and the concentration of Reg3γ in serum ($n=6$). *il-17d* was silenced in fish fed with SMC diet. **H** Survival rate of Nile tilapia against *A. hydrophila* infection of SM, SMC, and SMC + *il-17d* silenced fish ($n=3$ tanks, 10 fish per tank). **I** The gene expression of *il-17d* in the intestine ($n=6$). **J** the concentration of IL-17D in serum. **K** The gene expression of *reg3g* in the intestine ($n=6$). Data was expressed as mean ± SEM. SM, fish fed with soybean meal-based diet; SMC, fish fed with soybean meal-based diet supplemented with 3000 U/kg xylanase; SMC + dsRNA, *il-17d* silenced fish fed with soybean meal-based diet supplemented with 3000 U/kg xylanase. The significant differences between two group were presented at $P<0.05$ (*)

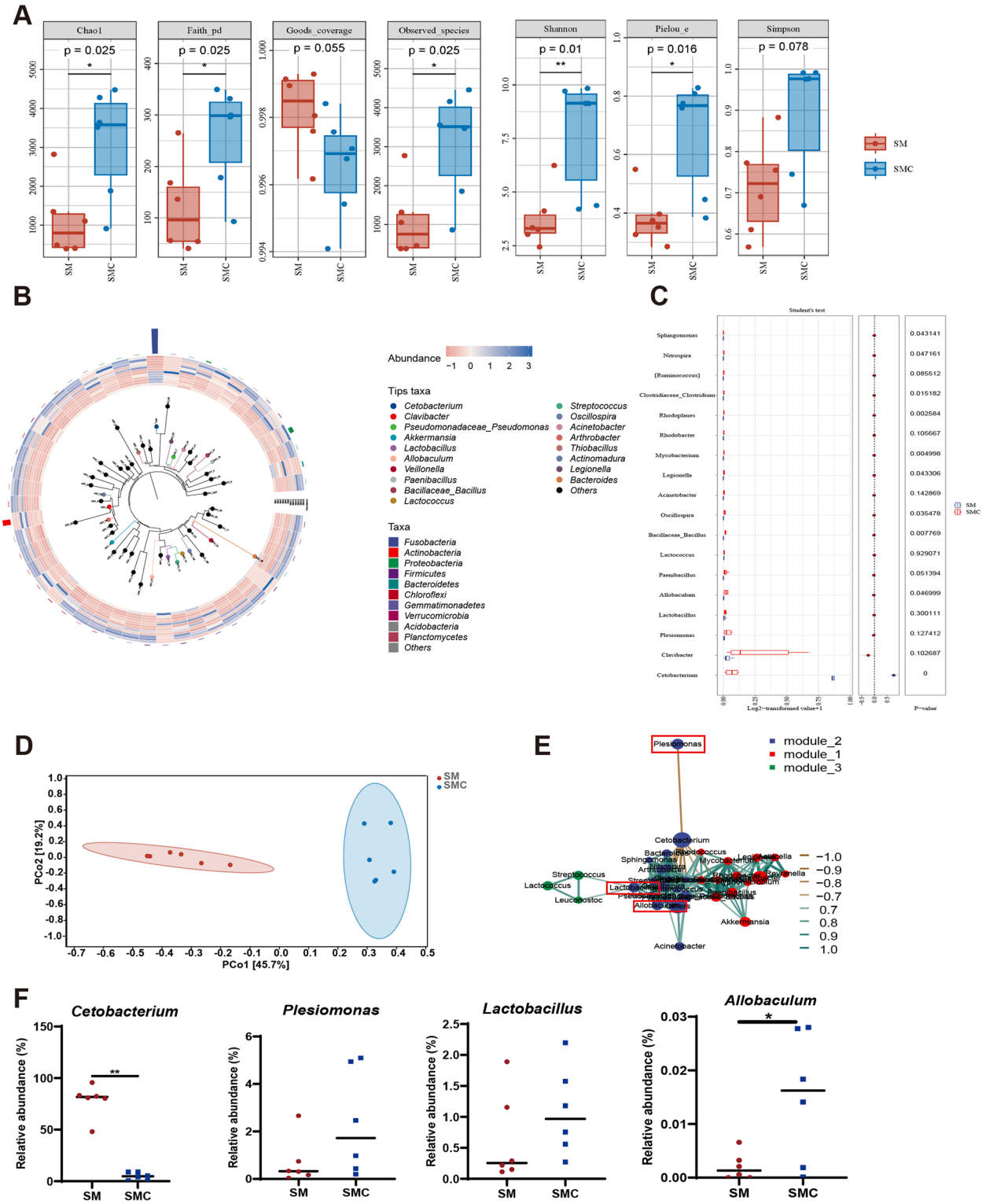

Fig. 2 The composition of intestinal microbiota by V3–V4 Illumina sequencing. **A** Alpha-diversity index of gut microbiota, including Chao1, Faith, Goods, Observed species, Shannon, Pielou, and Simpson, **B** intestinal microbiota composition in genus level, **C** difference analysis of 17 genera, **D** principal coordinates analysis (PCoA), **E** correlation network analysis based on spearman in genus, **F** the abundance of *Cetobacterium*, *Plesiomonas*, *Lactobacillus*, and *Allobaculum* ($n = 6$). Data was expressed as mean ± SEM. The significant differences between two group were presented at $P < 0.05$ (*) and $P < 0.01$ (**). SM, fish fed with soybean meal-based diet; SMC, fish fed with soybean meal-based diet supplemented with 3000 U/kg xylanase

sequencing analysis based on full length (approx. 1500 bp) of 16S rRNA gene was also conducted. For full-length 16S rRNA PacBio sequencing, an average of 37,894 reads was generated for each sample. There was no significant difference in the indices of Chao1, Faith, Goods, and Observed species between the SM and SMC groups (Fig. 3A). Xylanase administration significantly increased Shannon, Pielou, and Simpson indices (Fig. 3A). PacBio sequencing data set showed that the dominant phyla were Fusobacteria (70.62%), Bacteroidetes (8.52%), Proteobacteria (7.22%), and Firmicutes (3.37%). However, in the SMC group, Fusobacteria (46.95%), Proteobacteria (29.21%), Planctomycetes (4.43%), Actinobacteria (4.12%), and Chloroflexi (5.82%) were the top 5 phyla (Fig. 3B). The relative abundance of top 50 genera accounted for 75% in the

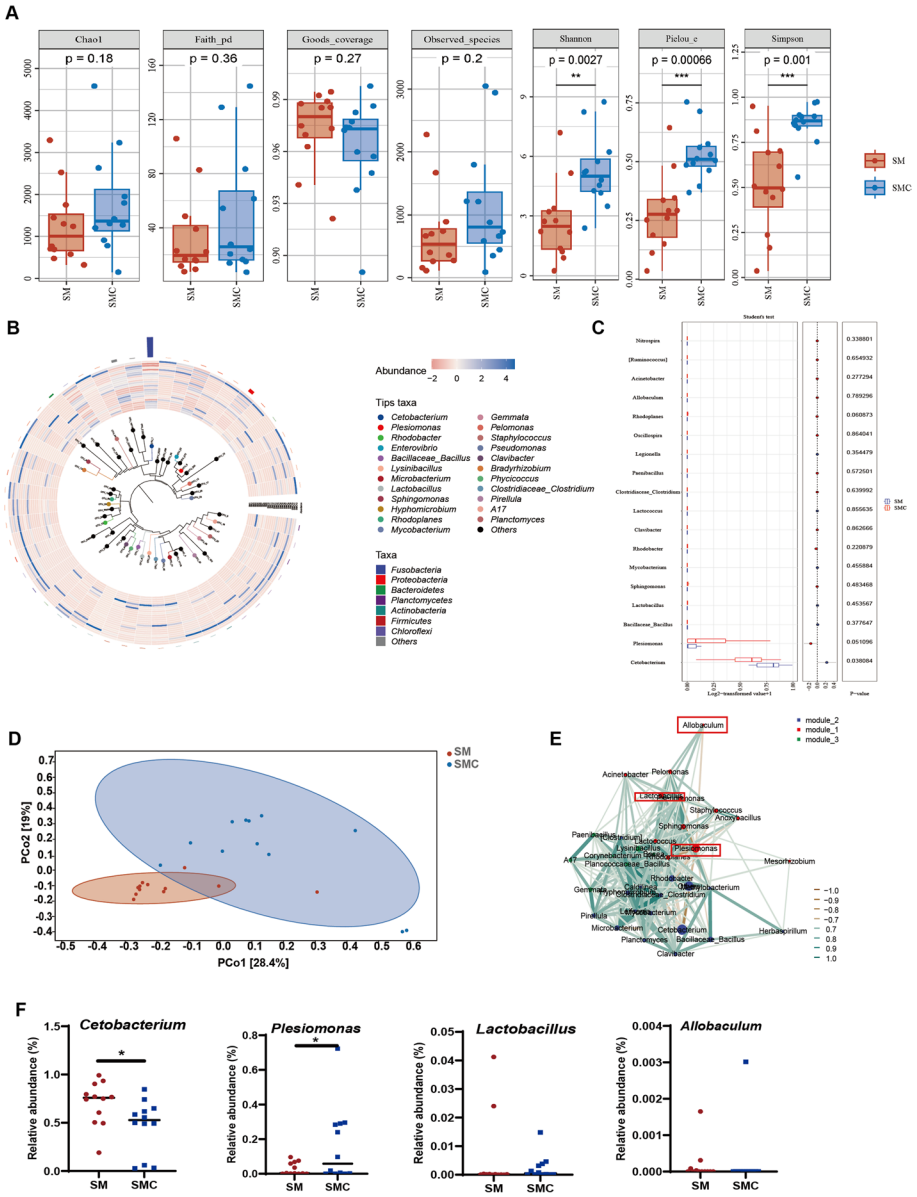

Fig. 3 The composition of intestinal microbiota by full-length 16S rRNA PacBio sequencing. **A** Alpha-diversity index of gut microbiota, including Chao1, Faith, Goods, Observed species, Shannon, Pielou, and Simpson, **B** intestinal microbiota composition in genus level, **C** difference analysis of 17 genera, **D** principal coordinates analysis (PCoA), **E** correlation network analysis based on spearman in genus, and **F** the abundance of *Cetobacterium*, *Plesiomonas*, *Lactobacillus*, and *Allobaculum* ($n = 12$). Data was expressed as mean ± SEM. The significant differences between two group were presented at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). SM, fish fed with soybean meal-based diet; SMC, fish fed with soybean meal-based diet supplemented with 3000 U/kg xylanase

PacBio sequencing. Consistent with Illumina V3–V4 sequencing, PacBio sequencing found that xylanase decreased the abundance of *Cetobacterium* (46.69%) and increased the abundance of *Plesiomonas* (16.32%) in the SMC group (Fig. 3C). PCoA explained 28.4% (PCo1) and 19% (PCo2) of the data in PacBio sequencing (Fig. 3D). The interaction of the top 50 genera was analyzed by correlation network analysis. The analysis indicated that the genus of *Plesiomonas, Lactobacillus*, and *Allobaculum* belong to same module_1 (Fig. 3E, F).

There were some differences in the relative abundance of gut microbiota based on these two sequencing methods, but the dominant bacterial members in two groups were similar. The dominant bacteria in the soybean meal group include Fusobacteria, Proteobacteria, Firmicutes, and Bacteroidetes, while those in the xylanase group consist of Actinobacteria, Proteobacteria, Chloroflexi, and Fusobacteria. Both sequencing methods indicated that supplementation of xylanase in soybean meal improved the α-diversity of gut microbiota and altered the gut microbial composition of Nile tilapia.

Xylanase directly promotes the growth of A. stercoricanis

Correlation analysis of both sequencing methods showed that the genera *Plesiomonas*, *Lactobacillus*, and *Allobaculum* belong to the same module, while *Akkermansia* was in a different module. Based on the results of correlation network analysis (Figs. 2E & 3E), three species — including *P. shigelloides*, *L. plantarum*, and *A. stercoricanis* — were selected for further analysis. *Akkermansia* was used as a control to validate the accuracy of the correlation analysis. Soybean meal was used as the carbon source in the bacteria culture medium, and the data showed that xylanase did not promote the proliferation of *P. shigelloides*, *L. plantarum*, or *A. muciniphila* in vitro (Fig. 4A, B, C). Compared with SM treatment, xylanase

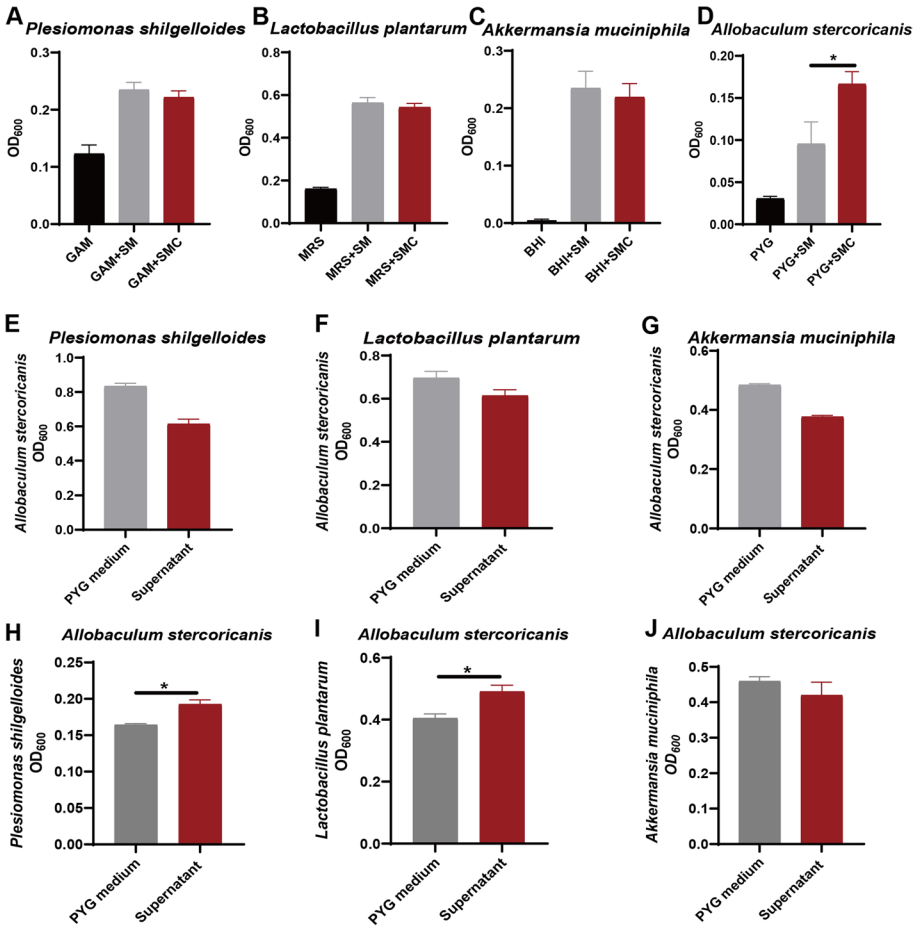

Fig. 4 Xylanase directly promotes the proliferation of bacteria. **A**, **B**, **C**, **D** The effects of xylanase on bacterial proliferation, including *P. shigelloides*, *L. plantarum*, *A. muciniphila*, and *A. stercoricanis*. **E**, **F**, **G** The effects of *P. shigelloides*, *L. plantarum*, and *A. muciniphila* on the growth of *A. stercoricanis*. **H**, **I**, **J** The effects of *A. stercoricanis* on the proliferation of *P. shigelloides*, *L. plantarum*, and *A. muciniphila*. Data was expressed as mean ± SEM ($n = 3$). The significant differences between two group were presented at $P < 0.05$ (*). SM, soybean meal; SMC, soybean meal supplemented with 3000 U/kg xylanase

significantly enhanced the growth of *A. stercoricanis* in vitro (Fig. 4D). Next, we used the supernatant of *P. shigelloides*, *L. plantarum*, and *A. muciniphila* to culture *A. stercoricanis*, and the results showed that these three bacteria did not promote the growth of *A. stercoricanis* (Fig. 4E, F, G). However, the supernatant of *A. stercoricanis* promoted the proliferation of *P. shigelloides* and *L. plantarum* (Fig. 4H, I), while it had no impact on the growth of *A. muciniphila* (Fig. 4J). These data suggested that xylanase directly promoted the growth of *A. stercoricanis*, which in turn enhanced the growth of *P. shigelloides* and *L. plantarum*.

Xylanase supplementation elevated the butyrate level and the expression of IL-17D

After feeding SMC, the concentrations of short-chain fatty acids (SCFAs) in the intestine of Nile tilapia were analyzed. Xylanase intervention did not influence the level of acetic acid and propionic acid (Fig. 5A, B) but significantly increased the concentration of butyric acid (Fig. 5C). Next, we detected the production of short-chain fatty acids by these bacteria, and the results showed that *Cetobacterium somerae* and *A. muciniphila* produced acetic acid and propionic acid, while *P. shigelloides* and *L. plantarum* only secreted propionic acid (Fig. 5D). The butyric acid is only detected in the culture supernatant of *A. stercoricanis* (Fig. 5D). The supernatant and pasteurized cells of *A. stercoricanis* were used to treat the primary intestinal cells. The results showed that the pasteurized cells did not influence the expression of *il-17d*, but the supernatant of *A. stercoricanis* significantly activated its expression level (Fig. 5E). In combination with the above results, we hypothesized that butyrate may be the key mediator through which xylanase enhances pathogen resistance in Nile tilapia.

To verify the immunoprotective effect of butyric acid, sodium butyrate was added to the soybean meal-based diet, and the challenge experiment showed that sodium butyrate reduced the mortality of Nile tilapia (Fig. 6A). In addition, sodium butyrate strongly activated the gene expression of *il-17d* and *reg3g* in the intestine and significantly elevated the concentration of IL-17D and Reg3γ in serum (Fig. 6B, C, D, E). The silence of *il-17d* in fish fed with soybean meal-based diet containing sodium butyrate (SB) diet decreased the survival rate of Nile tilapia when they were challenged with *A. hydrophila* compared with SB group (Fig. 6F). Consistent with the experimental results in xylanase supplementation, the

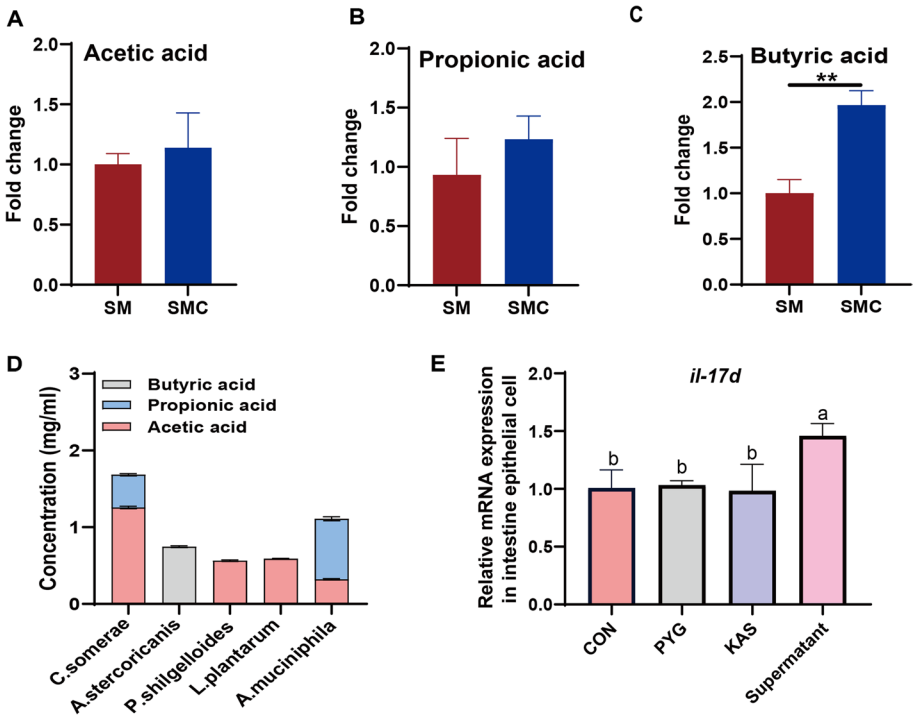

Fig. 5 Xylanase elevated the butyrate level in the gut. **A** Acetic acid. **B** Propionic acid. **C** Butyric acid. **D** The types of short-chain fatty acid in *C. somerae*, *A. stercoricanis*, *P. shigelloides*, *L. plantarum*, and *A. muciniphila*. **E** The effects of pasteurized bacteria (KAS) and supernatant of *A. stercoricanis* on the expression of *il-17d* in intestinal cells. Data was expressed as mean ± SEM ($n = 3$). The significant differences between two groups were presented at $P < 0.01$ (**) based on Student's test. Statistically significant results were expressed by lowercase letters (a, b, c) based on ANOVA with Tukey test. SM, soybean meal; SMC, soybean meal supplemented with 3000 U/kg xylanase

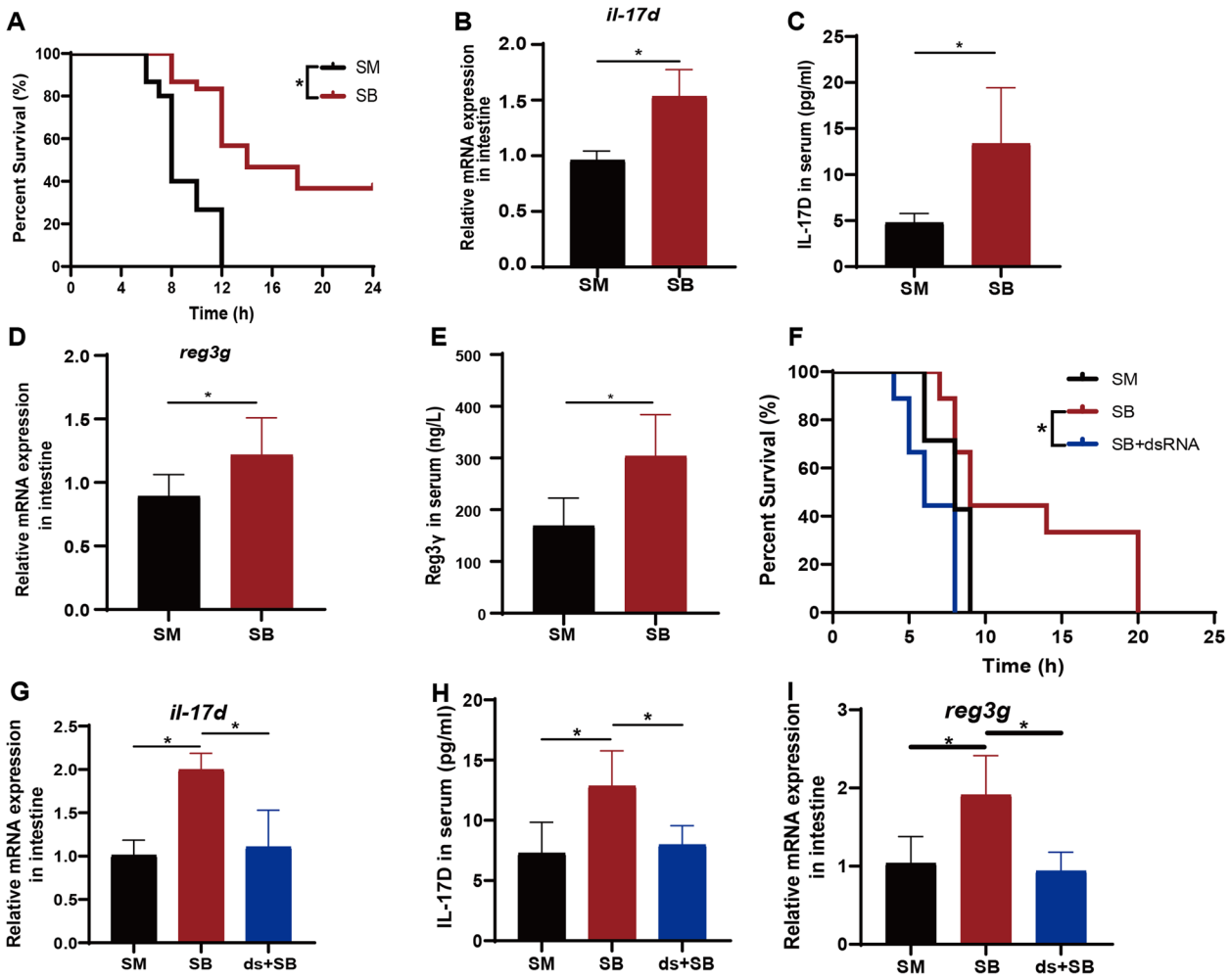

Fig. 6 Butyrate improved the pathogen resistance by activating the expression of IL-17D. **A** Survival rate of Nile tilapia against *A. hydrophila* infection of SM and SB group ($n=3$ tanks,10 fish per tank), **B** the gene expression of *il-17d* in the intestine ($n=6$), **C** the concentration of IL-17D in serum ($n=6$), **D** the gene expression of *reg3g* in the intestine ($n=6$), **E** the concentration of Reg3γ in serum ($n=6$), *il-17d* was silenced in fish fed with SB diet, and **F** survival rate of Nile tilapia against *A. hydrophila* infection of SM, SB, and SB + *il-17d* silenced fish ($n=3$ tanks, 10 fish per tank). **G** the gene expression of *il-17d* in the intestine ($n=6$), **H** the concentration of IL-17D in serum ($n=6$), and **I** the gene expression of *reg3g* in the intestine ($n=6$). Data was expressed as mean ± SEM. SM, fish fed with soybean meal-based diet; SB, fish fed with soybean meal-based diet supplemented with 40 mmol/kg sodium butyrate. ds + SB, fish fed with soybean meal-based diet supplemented with 40 mmol/kg sodium butyrate, and the *il-17d* was silenced. The significant differences between two group were presented at $P<0.05$ (*) and $P<0.01$ (**)

knockdown of *il-17d* inhibited the gene expression *il-17d* and *reg3g* in the intestine, and it also reduced the concentration of IL-17D and Reg3γ in serum (Fig. 6G, H, I). Hence, these finding suggested that sodium butyrate plays a crucial role in the immunoprotective effects of xylanase by activating the expression of *il-17d* and *reg3g*.

Butyrate increased the IL-17D production in intestinal epithelial cells

Given the differences in intestinal structure between fish and mammals, we aimed to identify the cellular source of IL-17D by isolating intestinal lymphocytes and intestinal epithelial cells. Flow cytometry analysis confirmed that sodium butyrate increased the number of intestinal cells expressing IL-17D in fish (Fig. 7A), while it had no effect on IL-17D expression in intestinal lymphocytes (Fig. 7B). In marked contrast, sodium butyrate elevated the number of IL-17D$^+$ intestinal epithelial cells (Fig. 7C) and significantly upregulated their *il-17d* expression (Fig. 7D). Consistent with the results in vivo, sodium butyrate activated the expression of *il-17d* and *reg3g* in intestinal epithelial cells ex vivo (Fig. 7E, F), indicating that intestinal epithelial cells are the primary source of IL-17D in Nile tilapia.

Butyrate promotes il-17d expression mainly by HDAC inhibitor

To explore how butyrate actives the expression of *il-17d*, two possible mechanisms, including activation of

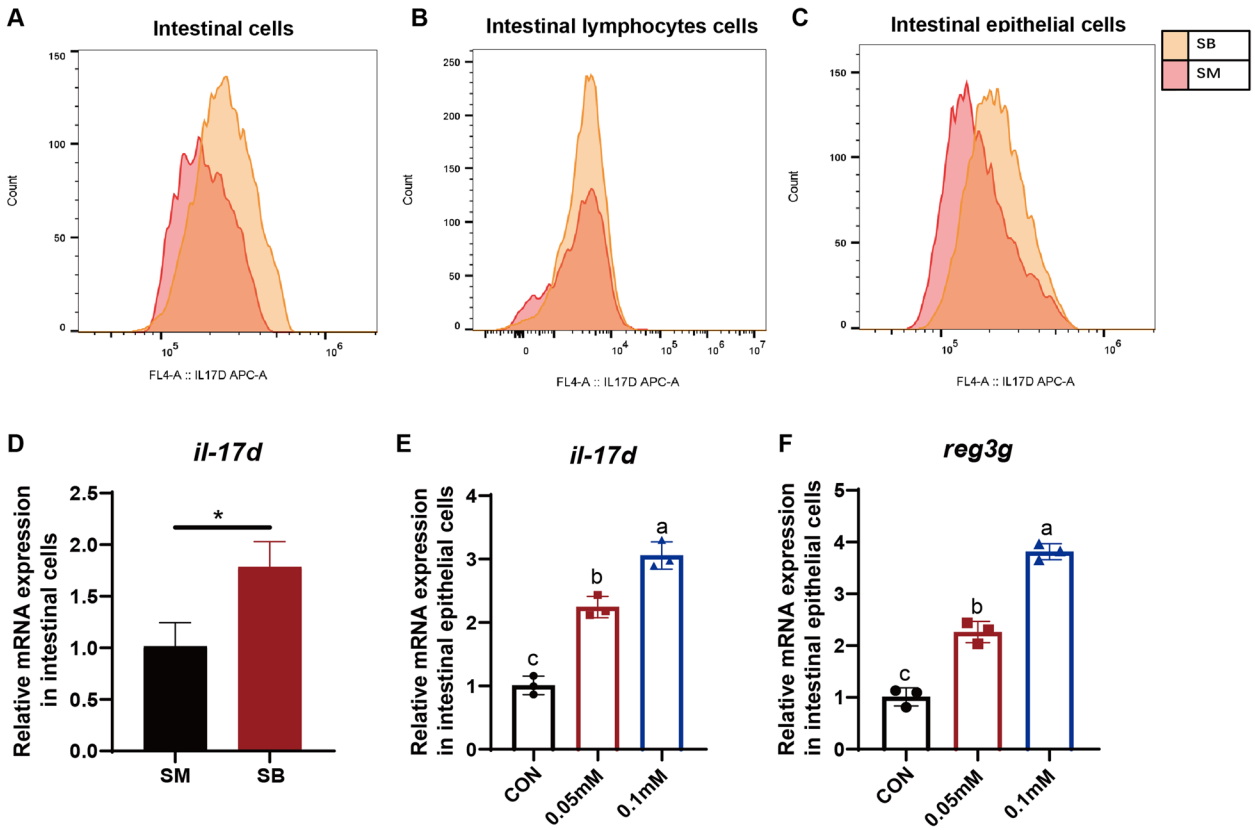

Fig. 7 Butyrate increased the IL-17D production in intestinal epithelial cells in vivo and in vitro. **A**, **B**, **C** Flow cytometry gating shows IL-17D analysis in viable intestine cells, lymphocytes cells, and epithelial cells from SM and SB fish, **D** the gene expression of *il-17d* in intestinal epithelial cells from SM and SB fish, the effects of sodium butyrate on the expression of **E***il-17d*, and **F** *reg3g* in primary intestinal epithelial cells. Data was expressed as mean ± SEM ($n = 3$). SM, fish fed with soybean meal-based diet; SB, fish fed with soybean meal-based diet supplemented with 40 mmol/kg sodium butyrate. The significant differences between two group were presented at $P < 0.05$ (*) based on Student's test. Statistically significant results were expressed by lowercase letters (a, b, c) based on ANOVA with Tukey test

G protein-coupled receptors (GPCRs) or suppression of histone deacetylation (HDAC), were investigated. The intestinal epithelial cells were treated with two GPCR inhibitors, and the results showed that inhibition of FFAR2 or FFAR3 had no effect on butyric acid-stimulated *il-17d* expression (Fig. 8A). The balance of acetylation of histones is regulated by the NAD + -dependent, silent information regulator 2 (SIRT) and HDAC families, which in turn can mediate cell proliferation, differentiation, and immunity. The SIRT inhibitor — nicotinamide (NAM) and the HDAC inhibitor — trichostatin (TSA) were used to treat intestinal epithelial cells, and the data showed that TSA promoted the gene expression of *il-17d* in intestinal epithelial cells as well as sodium butyrate, but not NAM (Fig. 8B, C). HADC activator ITSA was also added to intestinal epithelial cells, and the results showed that ITSA partially inhibit the expression of *il-17d* in sodium butyrate-treated cells (Fig. 8D). These results suggested that butyrate activated the expression of *il-17d* by inhibiting HDAC, and the increased pan-acetylation further confirms this finding (Fig. 8E).

The HDAC family contains many deacetylases, but it is unclear about which enzyme is involved in regulating the expression of *il-17d*. Then Biological General Repository for Interaction Datasets (BioGRID) predicted the interaction may exist between IL-17D and HDAC3. Consistent with the prediction, butyrate largely inhibited the protein expression of HDAC3 (Fig. 8F). To further confirm the relationship between IL-17D and HDAC3, HDAC3 was inhibited by the specific inhibitor of the class I histone deacetylase, RGFP966. The data reveled that the inhibition of HDAC3 significantly increased the expression of IL-17D in intestinal epithelial cells (Fig. 8G). To explore the link between butyrate and *il-17d* gene transcription, a luciferase reporter plasmid containing the *il-17d* promoter was constructed. The data showed that butyrate improved the transcription of *il-17d* in intestinal epithelial cells by inhibiting HDAC3 (Fig. 8H).

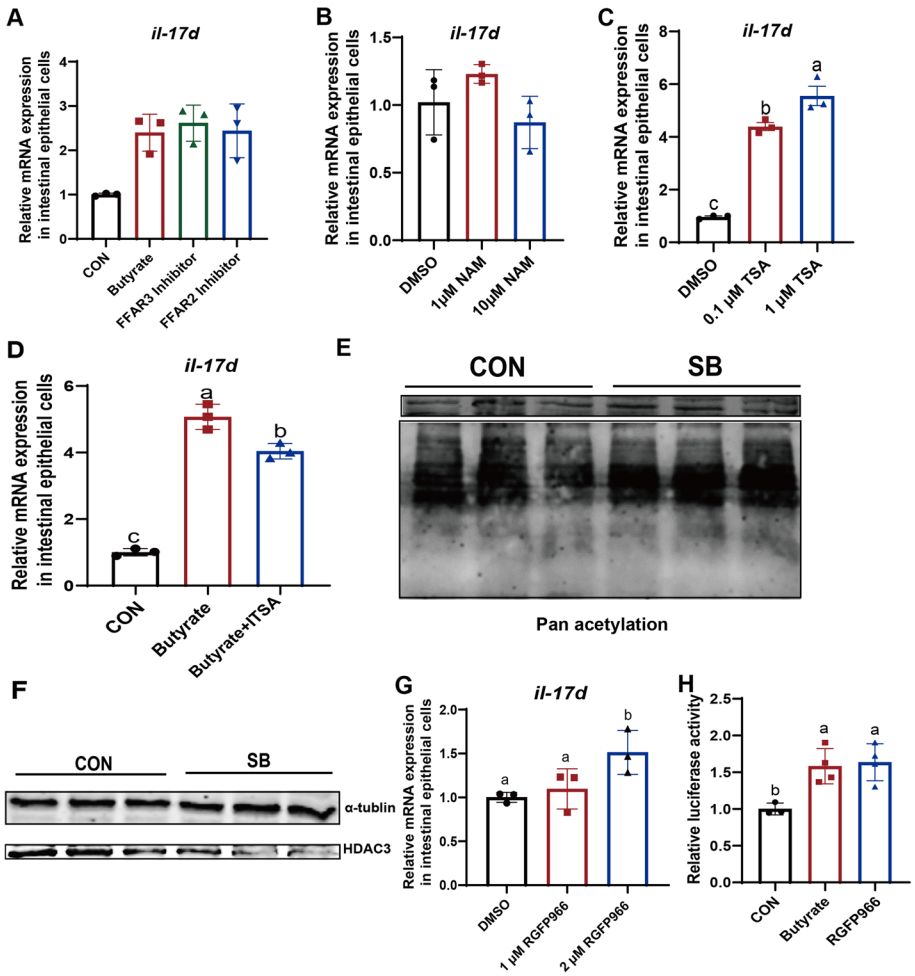

Fig. 8 Butyrate promotes *il-17d* expression mainly by HDAC inhibitor. **A**, **B**, **C**, **D** The effects of FFAR2 inhibitor, FFAR3 inhibitor, NAM, TSA, and ITSA on the *il-17d* expression of intestinal epithelial cells, **E** the effects of sodium butyrate on the pan-acetylation level of intestinal epithelial cells, **F** the protein expression of HDAC3, **G** the effects of RGFP966 (HDAC3 inhibitor) on the *il-17d* expression of intestinal epithelial cells, and **H** luciferase reporter plasmid pGL 4.10- *il-17d* -luc were transiently transfected into 293 T cells. Cells were treated with butyrate or RGFP966 for 12 h. Luciferase activities of untreated cells and butyrate or RGFP966-treated cells are depicted relative to the *Renilla* activities. Data was expressed as mean ± SEM ($n = 3$). SB, cells treated with sodium butyrate. FFAR2/3, free fatty acid receptor 2/3. NAM, nicotinamide. TSA, trichostatin A. ITSA, inhibitor of trichostatin. Statistically significant results were expressed by lowercase letters (a, b, c) based on ANOVA with Tukey test

Discussion

In the present study, the supplementation of xylanase in soybean meal-based diet enhanced the pathogen resistance of Nile tilapia. Mechanistically, dietary xylanase altered the intestinal microbiota composition and increased the concentration of butyrate. Surprisingly, sodium butyrate inhibits HDAC3 to activate the expression of IL-17D in intestinal epithelial cells, which subsequently boosted the production of Reg3γ, ultimately improving resistance against pathogens in Nile tilapia (Fig. 9).

Soybean meal-based diet decreased the survival rates of Nile tilapia, possibly due to the anti-nutritional factors it contains, such as non-starch polysaccharides [28, 29]. Exogenous addition of xylanase could mitigate the negative effects caused by non-starch polysaccharide (NSP). Supplementation of *Bacillus amyloliquefaciens* R8, which expresses the recombinant xylanase, enhanced the immunity of Nile tilapia challenged with *A. hydrophila* and improved lysozyme activity in the serum [30]. *Jin* et al. found that dietary xylanase decreased the morbidity of grass carp against with *A. hydrophila*. Meanwhile, supplementation of xylanase altered the gut microbiota composition and increased the gene expression of *hepcidin* in the intestine [23]. The above studies showed that xylanase could affect the expression of antimicrobial peptides and the intestinal microbiota, but the underlying mechanism remains

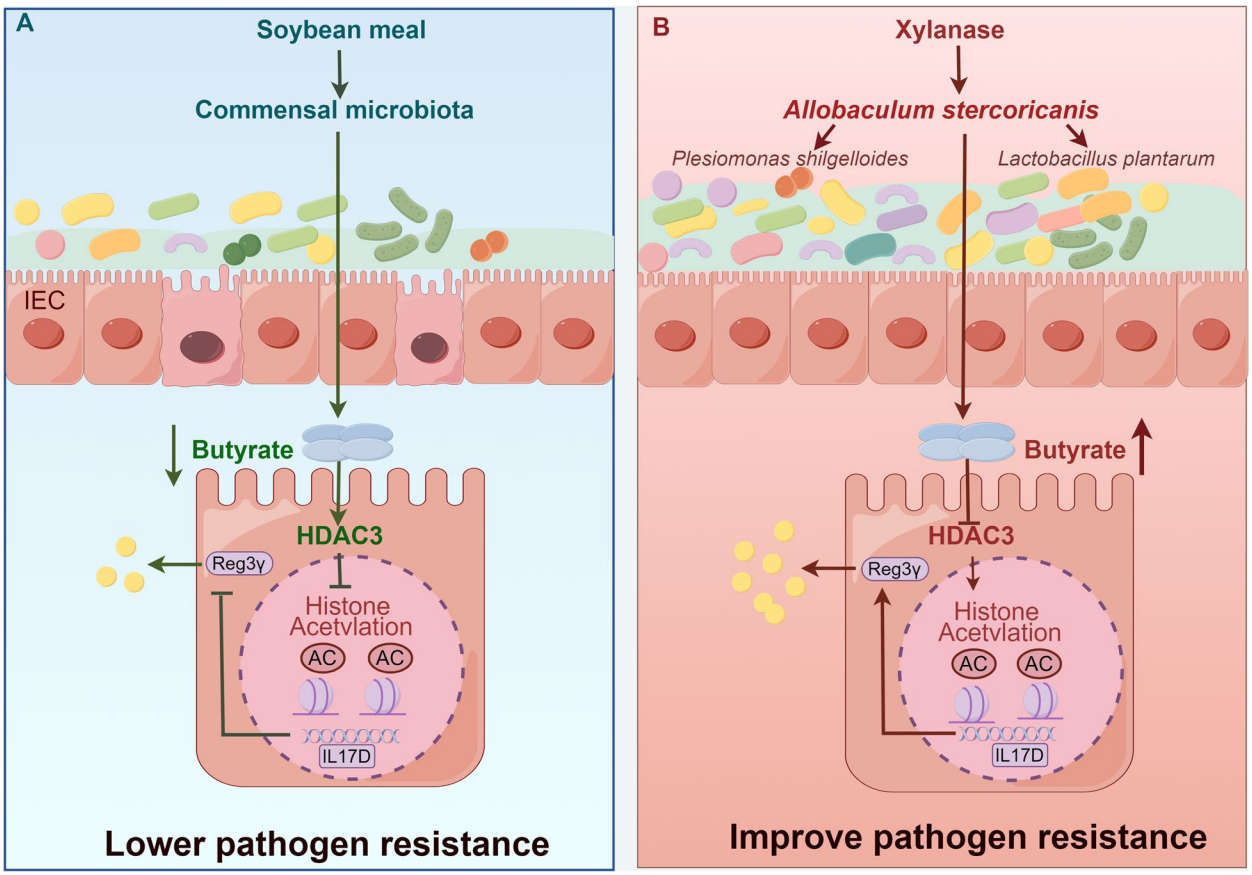

Fig. 9 Dietary xylanase altered the composition of the intestinal microbiota, notably promoting the proliferation of *A. stercoricanis*, which produces butyrate in vitro. Mechanically, butyrate activated the gene transcription of IL-17D in intestinal epithelial cells by inhibiting histone deacetylase activity. The increased expression of IL-17D could protect the Nile tilapia from pathogen infection

unclear. Our study revealed that xylanase boosted the resistance against *A. hydrophila* by increasing antimicrobial peptide, Reg3γ, which was regulated by IL-17D. IL-17D is one of the four IL-17 family members found in the genome of Nile tilapia. In mice, there are six members in IL-17 family including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [31]. In mammals, IL-17D plays an important role in antipathogenic responses and inflammation. Mice lacking *il-17d* were more susceptible to group A *Streptococcus* infection and had lower weight and higher bacterial burdens [32]. Overexpression of IL-17D could increase the recruitment of natural killer cells to reject tumor by stimulating monocyte chemotactic protein-1 (MCP-1) production from tumor endothelial cells [33]. IL-17D is also required for IL-22 production and regulates the expression of IL-22-dependent antimicrobial peptides- Reg3β and Reg3γ, which protect mice against colitis [34]. All these studies indicate that IL-17D plays an important role in the mammalian immune system. In Nile tilapia, we also found that fish with silenced IL-17D had lower expression of *reg3g* and were more susceptible to *A. hydrophila* infection, leading to lower survival rates. This suggests that the IL-17D-Reg3γ signaling axis is conserved in resisting pathogenic infection in fish as well as mammals [35]. Previous studies have found that Nrf2 could modulate the expression of IL-17D by directly binding to the region of *il-17d* promoter and oxidative stress [36]. However, there was no change in the expression of *nrf2* in our study (data not shown), suggesting that a new mechanism is involved in regulating the expression of IL-17D.

Our results indicated that dietary xylanase altered the gut microbiota composition of Nile tilapia. The intestinal microbiota fermented the oligosaccharides to generate SCFAs. Consistent with previous studies [23], our results found that xylanase increased the content of butyrate in the gut. There are two potential mechanisms by which butyrate exerts its function in mammals. First, butyrate activates multiple GPCRs, including Gpr43 and Gpr41, in intestinal epithelial cells to promote immune responses [37]; second, it acts as a histone deacetylase inhibitor to regulate immune

cytokines [38, 39]. Histone deacetylases (HDACs) are involved in a wide range of physiological processes, including transcriptional regulation, cell proliferation, metabolism, and immune response [40]. It has been found that butyrate promoted the production of IL-22 through GPR41 and inhibited histone deacetylase (HDAC) in $CD4^+$ T cells [41]. In the present study, inhibition of Gpr43 or Gpr41 did not influence the expression of IL-17D in butyrate-treated fish; instead, butyrate elevated the pan-acetylation level of intestinal epithelial cells to increase the expression of IL-17D. Our data indicated that HDAC family, instead of SIRT family, regulates the expression of IL-17D. It has been reported that sodium butyrate increased the IL-5 mRNA expression by directly activating on the IL-5 promoter, and this activation is related to the inhibition of HDAC3 in Jurkat cells [42]. In the present study, exogenous luciferase activity experiment confirmed that butyrate or HDAC3 inhibitor activated the promoter of IL-17D in intestinal epithelial cells. In general, our research demonstrates for the first time that butyric acid inhibits the transcription of IL-17D in intestinal epithelial cells by suppressing HDAC3 activation (Fig. 9). We revealed that intestinal epithelial cells in fish play a crucial role in immune responses. Additionally, it demonstrates the involvement of deacetylases in regulating the transcription of IL-17D.

In the present study, xylanase significantly increased the concentration of butyrate in the intestine. The immune-protective effects of butyric acid have been established in numerous mammalian hosts [43]. However, there has been limited exploration of the mechanisms by which butyric acid regulates the immune status of fish. For adaptive immunity, sodium butyrate improved host immunity by decreasing T-regulatory cells and increasing natural killer T cells and T-helper 17 cells in mice [44]. Conversely, sodium butyrate acts on innate immunity by reducing the recruitment of neutrophils and pro-inflammatory macrophages to wounds in zebrafish [45]. Most of these studies focus on the effects of butyric acid on immune cells of host, and little attention is paid to how butyric acid regulates the immune function of intestinal epithelial cells. Actually, intestinal epithelial cells could secrete many interleukins to regulate intestinal inflammation, such as IL-10, IL-8, IL-17C, and IL-25, and a number of chemokines [46, 47]. In fish, butyrate is mostly considered as an energy source for intestinal epithelial cells [48], but little attention was paid to the immune function of intestinal epithelial cells. In our study, we observed that butyrate activated the expression of IL-17D in epithelial cells, rather than immune cells. Like mammals, fish intestinal epithelial cells can secrete IL-17D [34]. These findings suggested that although the intestinal structure of fish differs from that of mammals, the immune function of intestinal epithelial cells is conserved.

We analyzed how xylanase modulated gut microbiota in Nile tilapia. Currently, microbial community profiling was sequenced by 16S rRNA gene amplicons on Illumina MiSeq. However, many bacterial species have similar variable region sequences which makes it difficult to distinguish these bacteria. The longer reads performed by Pacific Biosciences (PacBio) sequencing platform can give better-quality phylogenetic resolution [49]. In our work, V3–V4 Illumina sequencing indicated the xylanase administration increased the abundance of *Lactobacillus* and *Allobaculum*, while PacBio sequencing only found the abundance of *Plesiomonas* was increased in xylanase group. Similar to this result, previous studies also found that the abundance of *Lactobacillus* detected by the two sequencing methods was different [50]. Actually, the advantages and disadvantages of Illumina and PacBio sequencing are complementary [51]. High-throughput and high-precision short-read data are used to correct errors in long-read data, reducing the volume of longer reads with higher error rates and minimizing the presence of longer but erroneous data. Therefore, we combined two sequencing methods for bacterial community analysis. The correlation network analysis indicated that the genus of *Plesiomonas*, *Lactobacillus*, and *Allobaculum* belong to the same module. Although many studies have reported that xylanase can improve gut microbiota composition and promote the proliferation of beneficial bacteria [23, 52], the relationship between xylanase and the proliferation of these bacteria remains unclear. In the present study, the in vitro experiment results showed that xylanase directly promoted the growth of *A. stercoricanis*, which in turn enhanced the growth of *P. shigelloides* and *L. plantarum*. In mammals, metabolic interactions between bacteria can enhance gut microbiota stability. For example, *B. vulgatus* can promote the growth of *A. muciniphila* by degrading mucin, thereby improving host health [53]. Our results confirm the presence of cross-feeding phenomena in the fish gut, which helps maintain gut microbiota homeostasis. Furthermore, these demonstrated that the supernatant of *A. stercoricanis* can produce butyric acid and promote the expression of *il-17d*. These results revealed a complex interplay of microbial communities in the gut of Nile tilapia, influencing the host's immunity.

Conclusion

In the present study, we found that dietary xylanase altered the intestinal microbiota composition and improved the butyrate level in the gut. Mechanically, butyrate activated the gene transcription of IL-17D in intestinal epithelial cells by inhibiting histone deacetylase activity. The increased expression of IL-17D could protect the Nile tilapia from pathogen infection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01934-6.

Supplementary Material 1: Supplementary methods: Supplementary material 1.1. The isolation of *Lactobacillus plantarum* and *Plesiomonas shigelloides*. Supplementary material 2.2. The sequence of *il-17d* promoter. Supplementary figures: Figure S1. The experimental design of xylanase addition experiment. Figure S2. The experimental design of sodium butyrate addition experiment. Figure S3. The expression of*il-17d* after dsRNA silencing. Figure S4. The effects of xylanase on immune genes in intestine. (A) The gene expression related to immune responses in intestine by transcriptome analysis (n=4). (B) the gene expression of *il22* in intestine (n=6). Data was expressed as mean ± SEM. SM, fish fed with soybean meal diet; SMC, fish fed with soybean meal diet supplemented with 3000 U/kg xylanase. The significant differences between two group were presented at $P< 0.05$ (*). Supplementary tables: Table S1. Ingredients of the experimental diets. Table S2. Ingredients of the experimental diets. Table S3. Primer sequences used in qRT- PCR analyses.

Supplementary Material 2.

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Authors' contributions
We thank all authors for their contribution to the present study. MLZ and XYS designed the research. TW, NNZ, FFD, ZZH and ZYD conducted all experiments. TW analyzed the data. MLZ provided assistance in analyzing data. TW wrote the manuscript. MLZ and JG-V revised the manuscript. MLZ had primary responsibility for the final content. All authors read and approved the final manuscript.

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Data availability
The sequences are available in the Sequence Read Archive (SRA) under the BioProject accession number PRJNA1119640, PRJNA891636 and PRJNA1119706.

Declarations

Ethics approval and consent to participate
All animal experiments conducted at East China Normal University were approved by the Committee on the Ethics of Animal Experiments and performed according to the Management Rule of Laboratory Animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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