Fish and Shellfish Immunology 86 (2019) 53–63

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of *Lactococcus lactis* subsp. *lactis* JCM5805 on colonization dynamics of gut microbiota and regulation of immunity in early ontogenetic stages of tilapia

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ARTICLE INFO

Keywords:
Lactococcus lactis subsp. *lactis* JCM5808
Nile tilapia
Gut microbiota
IFN-α
Pathway

ABSTRACT

The administration of probiotics during early ontogenetic stages can be an effective way to manipulate the gut microbiota of animals. Specifically, the administration of probiotics can enhance gut-colonization success and regulate the immune response. In this study, the effects of early contact with probiotic *Lactococcus lactis* subsp. *lactis* JCM5805 on the gut microbial assembly of larvae Nile tilapia were examined. The effects of JCM5805 on *IFNα* expression through the TLR7 and TLR9-dependent signal transduction pathway as well as larval disease resistance were studied. Three days postfertilization, embryos were randomly allocated into nine 30 L tanks with a concentration of 20 eggs L^{-1}. Triplicate tanks were performed for each treatment. Treatments included a control group (C), a low probiotic concentration group (T1), where JCM5805 was added to the water at 1×10^4 cfu ml^{-1}, and a high probiotic concentration group (T2), where JCM5805 was added to the water at 1×10^8 cfu ml^{-1}. Probiotics were administered continuously for 15 days. qPCR was used to analyze transcript levels of the *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* genes using RNA extracted from whole embryos on day 5 and 10, and from the intestine of larvae on day 15. Transcription of these genes was also measured in the intestine, liver and spleen of larvae one month after the cessation of probiotic administration. The results showed that *MyD88* and *IRF7* were significantly elevated on days 5 and 10 in the T2 group. *TLR9* and *IFNα* were also significantly elevated on days 5, 10 and 15 during probiotic application of T2 ($P < 0.05$). One month after the cessation of probiotics administration, no significant difference was observed in the expression of these genes ($P > 0.05$). The larvae were fed probiotics for 15 days and were infused with *Streptococcus agalactiae* strain WC1535 at a final concentration of 1×10^6 cfu ml^{-1}. The survival rate of T2 was significantly higher than that of the C group ($P < 0.05$). Microbial characterization by Illumina HiSeq sequencing of 16S rRNA gene amplicons showed the significantly higher presence of JCM5805 in the guts of T2 after 15 days of probiotic continuous application. Although JCM5805 was below the detection level after the cessation of probiotic for 5 days, the gut microbiota of the exposed tilapia larvae in T2 remained clearly different from that of the control treatment after the cessation of probiotic administration. These data indicated that a high concentration of the probiotic strain JCM5805 upregulated the expression of *IFNα* via the *TLR7/TLR9-Myd88* pathway and enhanced disease resistance of larvae. JCM5805 was only transiently detected and thus was not included in the stable larval microbiota. The early microbial exposure of tilapia larvae affects the gut microbiota at later life stages. However, whether the upregulation of related genes is related to the presence of JCM5805 strain in the intestine requires further verification.

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https://doi.org/10.1016/j.fsi.2018.11.022
Received 2 October 2018; Received in revised form 5 November 2018; Accepted 7 November 2018

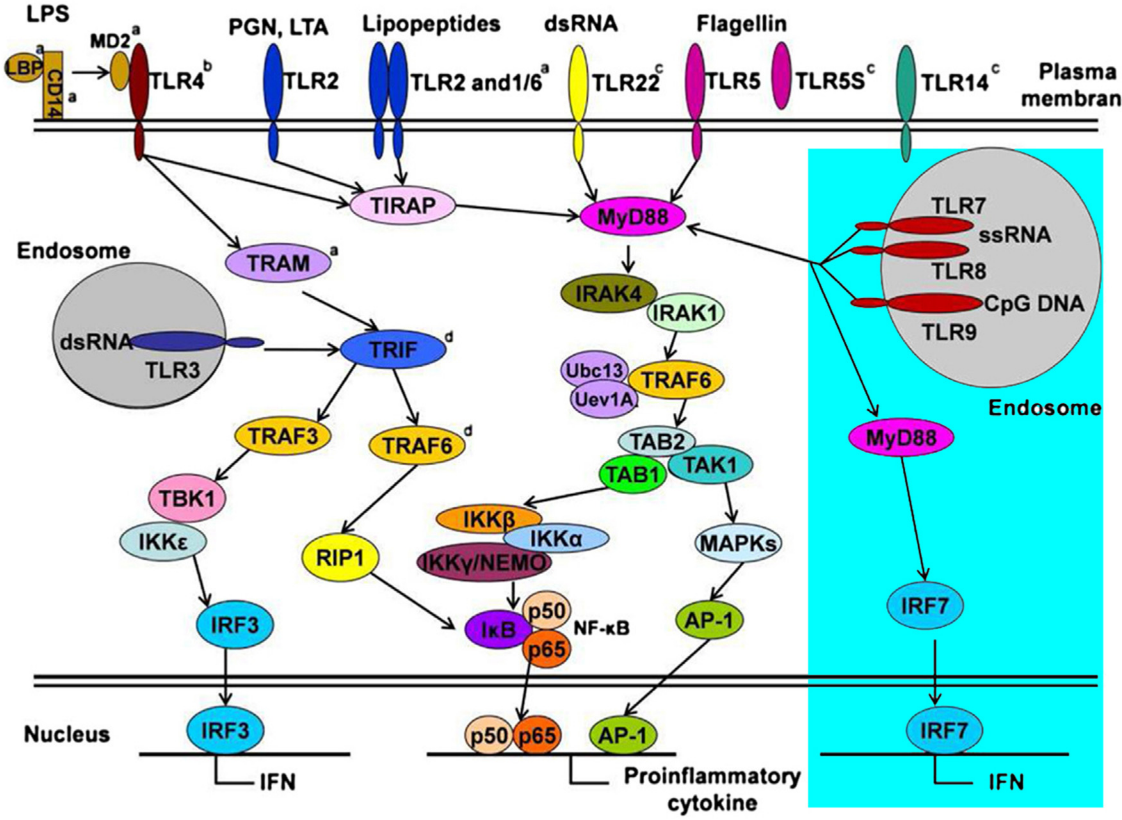

Fig. 1. TLR signaling pathways. a: molecules had not been found in fish (LBP, MD2, CD14, TRAM, TLR6). b: TLR4 was present in some but not all fish species. c: nonmammalian TLRs in fish (TLR5S, TLR14, TLR22). d: In mammals, TRIF interacts with TRAF6 and RIP1. However, TRIF fails to bind to TRAF6 in fish. Thus, NF-kB activation is dependent upon its interaction with RIP1 through an unknown mechanism. Modified from Ref. [23]. The green frame represents the gene in target signal pathway for this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
List of the reference and target gene primers used for qPCR.

Target gene	Primers	Oligonucleotide (50-30)	References (NCBI accession no.)	Product size (bp)	Ta (°C)	Efficiency
TLR7	F	ACGCTCACCATCAACCATATTCCG	XM_019352834.1	137	60	2.00
	R	ACACTCTTCAGGCACATCTTGTCC				
TLR9	F	CCATGGCTAAACATCTCTGCAC	XM_005477981.3	117	60	2.01
	R	GTCCAGGAAGGTAAAATTGACTC				
MyD88	F	GCCTGTGACTTTCAGACCAAG	NM_001311322.1	141	60	1.99
	R	CCGGGTGTAGTCACAAATGGT				
IRF7	F	CTTGGGAGGACGCTGACATTAT	XM_019361946.1	134	60	2.01
	R	TGGTTGCTCCTCTGTTGACTGG				
IFNα	F	AAGAATCGCAGCTCTGCACCATG	XM_019362701.1	134	60	1.98
	R	TGTGTCGTATTGCTGTGGCTTCC				
β-Actin	F	CAGGGAGAAGATGACCCAGA	XM_003443127.4	169	60	1.99
	R	CAGGGCATAACCCTAGTAGA				

1. Introduction

A vast number of microbial cells, approximately ten times more than host cells, reside in vertebrate and mammal gastrointestinal tract (GIT) and have been proven to maintain and modulate the balance of gut environment [1]. However, compared with terrestrial animals that undergo embryonic development within an amnion, fish larvae are released into the water at an early ontogenetic stage before their digestive tract and immune system have fully developed [2]. The fish gut microbiota is dependent on the aquatic environment. Use of probiotics in aquaculture is particularly effective during early ontogenetic stages and help prevent large mortalities that are commonly observed [2].

Probiotics are live microorganisms that confer several beneficial effects to hosts when consumed in adequate quantities [3,4]. These benefits can include enhanced immunity, improved digestion and protection from pathogens. In aquaculture, probiotics reduce infections caused by bacterial pathogens [5] and have been successfully used as immunostimulants [6] and growth promoters [7]. Probiotics are recognized as an effective way to modulate the intestinal microbiota. However, the ability of a probiotic strain to survive and successively proliferate in the gut after the cessation of probiotics administration is dependent on both the host and probiotic strain. The mode of administration, such as through water or feed, as well as the duration of administration, highly impact the survival and proliferation of the probiotic strain [7]. Some probiotic strains often only colonize the gut transiently and quickly fall below detection limits [2,8]. Current data indicates colonization dynamics of fish gut microbiota remain largely stochastic and are affected by elements of the gut habitat such as physiology and anatomy as well as host genotype [9–11]. Giatsis et al. suggested that early probiotic contact contributed to the subsequent

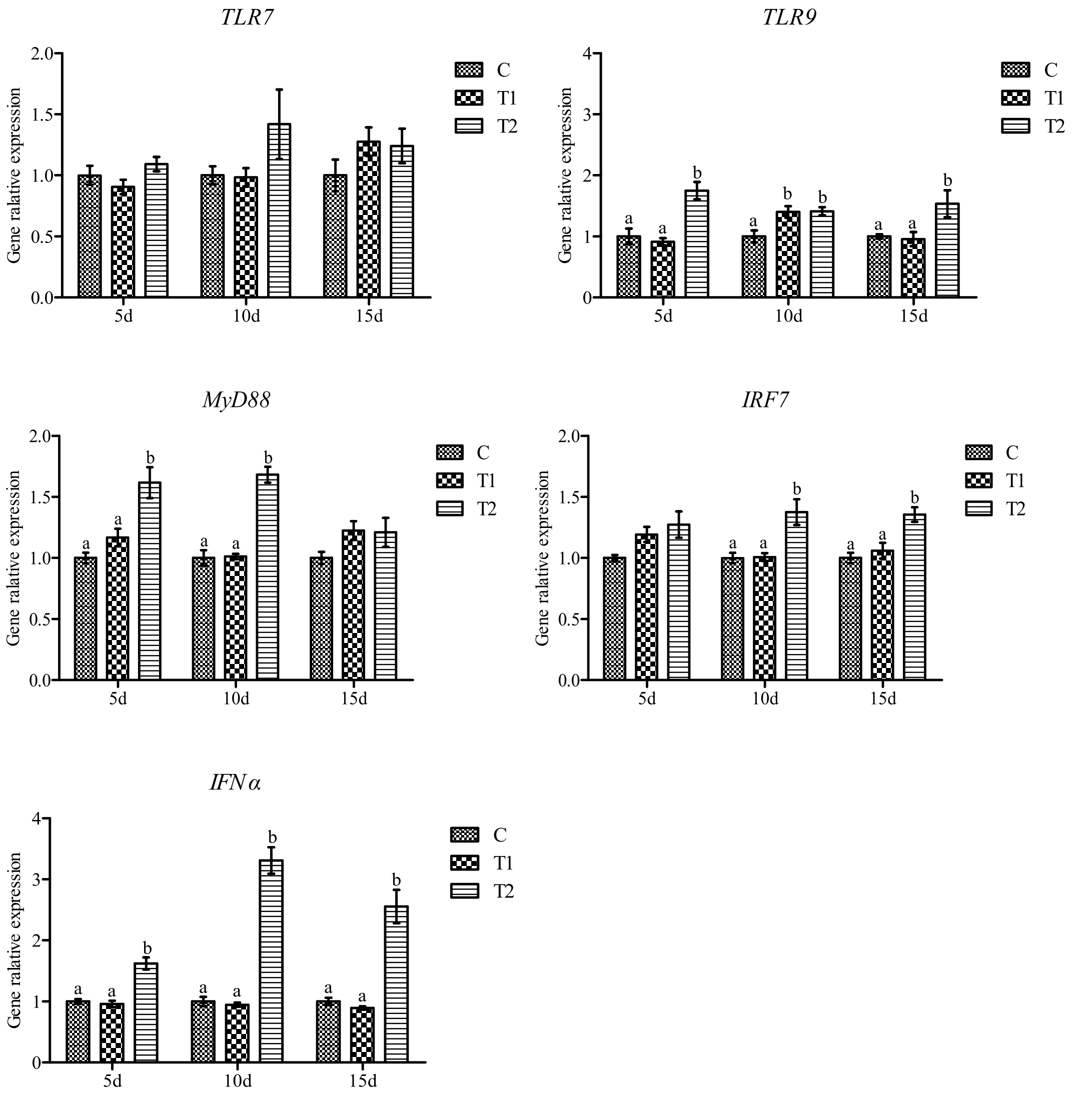

Fig. 2. Effects of JCM5805 on expression of *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* during embryonic development. The expression values were normalized to those of ATCB. The data are expressed as the mean fold change (means ± SE, n = 3) 5 days after administration of JCM5805. Bars with different letters indicate significant differences ($P < 0.05$). C: control group; T1: JCM5805 was added to the water at a concentration 1×10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration 1×10^8 cfu ml^{-1}.

observation of low interindividual variation of tilapia larvae [12]. Furthermore, tilapia larvae fed with different sludge-based microbial diets developed distinct gut microbiota, although all larvae also shared many common species [13]. In newly hatched chicks, it is possible to steer gut microbiota by feeding bacterial diets such as caecal inocula, which lead to the development of distinct communities [14].

The application of lactic acid bacteria (LAB) as feed additives instead of antibiotics or vaccine treatments is widely recommended in aquaculture. LAB can improve microfloral balance in the host intestine and enhance the defensive barrier against pathogens [15,16]. Due to food safety concerns, the concept of using probiotics derived from the human or food industry has been received with great interest in aquaculture. Some human-derived probiotics have been applied to promote growth and control infectious diseases in aquaculture [17,18]. The probiotic bacterium *Lactobacillus rhamnosus* GG (LGG) ATCC 53103, which is fit for human consumption, was originally used to promote general health status of humans and to prevent some diseases such as diarrhea and allergies [19,20], has been widely applied to control infections by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in rainbow trout [21] and *Edwardsiella tarda* and *Streptococcus agalactiae* in tilapia [17,18]. The supplementation of *L. rhamnosus* GG conferred an advantage in promoting intestinal structure and mucosal immunity of tilapia [22]. Our previous study indicated that use of JCM5805 can promote the growth, enhance both the immune status and disease resistance, and affect the gut microbiota of juvenile Nile tilapia [8]. Historically, JCM5805 has been primarily used in mammals [23,24]. *L. lactis* JCM5805 is a unique LAB that can directly activate plasmacytoid dendritic cells (pDCs) in mice and humans [23,25]. pDCs express two distinct types of Toll-like receptors (TLRs), TLR7 and TLR9 [26], which induce the production of type I interferons (IFNs) in a MyD88-dependent manner [27,28]. These IFNs act in the innate immune system as the first line of defense against viral infections. This signaling pathway also exists in aquatic animals [29].

Previous studies found that JCM5805 could proliferate in the gut of juvenile tilapia but did not colonize it [8]. In this research, the impact of early microbial contact with tilapia embryo and larvae on the gut microbial assembly during later ontogenetic stages was investigated. Is it possible that administration of JCM5805 to fish larvae early in life, when the gut microbiota is still developing, could enhance gut-colonization success and lead to the development of distinct gut communities? Would the gut community remain colonized even after the

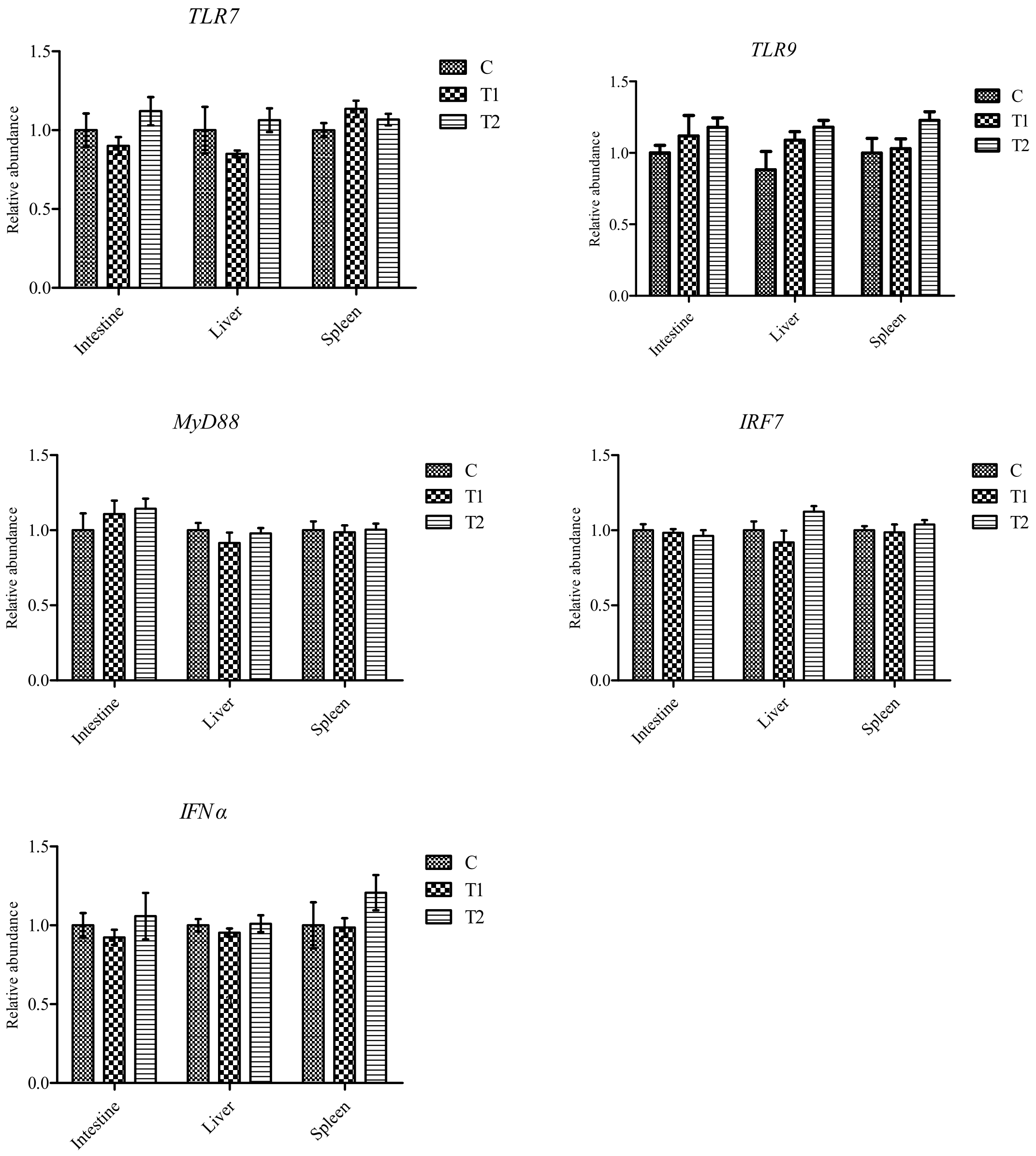

Fig. 3. Levels of *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* mRNA in the intestine, liver and spleen of tilapia 1 month after the cessation of probiotic administration. The expression values were normalized to those of ATCB. The data are expressed as the mean fold change (means ± SE, n = 3). Bars with different letters indicate significant differences ($P < 0.05$).

cessation of probiotic administration when the fish are exposed to conventional culture conditions? This research also focuses on the pathway through which TLR7 and TLR9 induce the production of type I IFNs in a MyD88-dependent manner, as well as the disease resistance of tilapia to *Streptococcus agalactiae*.

2. Materials and methods

2.1. Probiotic conditions

JCM5805 was purchased from the Japanese Collection of Microorganisms (JCM). Cultures of JCM5805 were grown at 30 °C for 48 h in Brain Heart Infusion Broth (BHI) (OXOID) according to the manufacturer's instructions. The cultures were centrifuged (Beckman Coulter, AK, USA) at 5000 g for 5 min. The pellets were washed twice with sterile distilled water, lyophilized and suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM NaH_2PO_4, 1.8 mM KH_2PO_4, pH 7.4). The number of the bacterial cells in each suspension was determine by turbidimetry.

2.2. Fish husbandry and administration

The experiment was conducted under normal conditions. Water conditions remained within the following ranges: 27 ± 0.5 °C, pH 6.5–7.1, dissolved oxygen > 6.1 ± 0.5 mg L^{-1}, NH_4^+-N < 0.5 mg L^{-1} and NO_2—N < 0.05 mg L^{-1}. Three days postfertilization (dpf), the eggs were randomly allocated to nine 30-L tanks (triplicate tanks per treatment) at a concentration of 20 eggs L^{-1}. Treatments included a control group (C), a low probiotic concentration group (T1) and a high probiotic concentration group (T2). Optical density (OD600) was used to adjust the JCM5805 cell density of the low and high probiotic-treat groups to 1×10^4 cfu ml^{-1} (T1) and 1×10^8 cfu ml^{-1} (T2), respectively. In the probiotic tanks, the water containing JCM5805 was replaced daily. Within probiotic groups, probiotic bacteria were supplied for 15 consecutive days (dpf 4–18); the larvae from these probiotic treatments were subsequently raised for another month (dpf 19–48) under the same normal conditions as the control group.

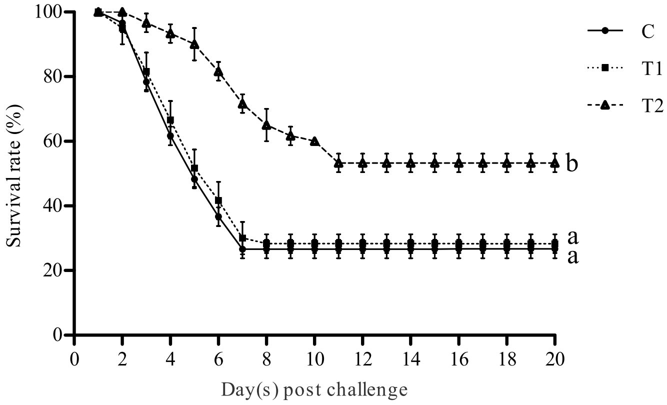

Fig. 4. Cumulative survival rates of tilapia challenged by injection of *S. agalactiae* after administering either basal diet or basal diet with probiotics for six weeks (means ± S.E.). Different letters denote significant differences ($P < 0.05$). C: control group; T1: JCM5805 was added to the water at a concentration 1×10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration 1×10^8 cfu ml^{-1}.

2.3. Expression of genes involved in target signaling pathway

The target signaling pathway related genes were *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* (Fig. 1) [29].

2.4. RNA extraction and cDNA synthesis

To analyze gene expression during embryonic development after probiotic administration, approximately 45 tilapia embryos and larvae were randomly sampled 5, 10 and 15 days after probiotics treatment. A total of 9 RNA samples were extracted for each treatment, with 3 samples taken for each time point; RNA was extracted from the entire sample on the 5th and 10th day, and from the whole gut of the larvae on the 15th day. After one month following the cessation of probiotics administration, the liver, spleen and mid-intestine of juveniles were also selected for RNA extraction. Total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen Biotech, ER501, Beijing, China), and RNA quality was analyzed by visualization on a 1.2% agarose gel. RNA was dissolved in 120 μl RNase-free water and stored at −80 °C until use. cDNA was synthesized for quantitative reverse transcription PCR (q-PCR) using the TransScript cDNA Synthesis SuperMix (TransGen Biotech, AT301, Beijing, China) according to the manufacturer's instructions.

2.5. Quantitative real-time PCR (qPCR)

Plasmids were constructed and used as external standards for each target gene. Briefly, primers (Table 1) were designed to amplify specific segments of the *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* genes. β-actin gene was chosen as the internal standard. PCR was performed using a cDNA template synthesized from total RNA from the liver using PrimeScript™ 1st-strand cDNA Kit (TaKaRa). Bands of the desired size were excised, purified and subcloned into the pMD19-T vector for sequencing. The purified plasmids were quantified with a Biophotometer using serially diluted standard samples ranging from 10^{-1} pmol/L to 10^{-8} pmol/L. All standard curves exhibited correlation coefficients higher than 0.99.

Real-time PCR analysis was performed with a LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland) using the Power SYBR Green Master Mix (Applied Biosystems). Reactions were performed in 20 μl volumes containing 1 μl cDNA template, 10 μl Power SYBR Green Master Mix, 0.3 μl forward and reverse primers (10 pmol/L) and 8.3 μl of ddH2O. The PCR parameters started with 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. A denaturing step of 15 s at 95 °C was added after the amplification step. To verify that a single PCR product was generated at the end of the assay, a melting curve analysis was performed over a range of 60–95 °C. Each assay was performed in triplicate. The negative controls in the real-time PCR analysis included a no-cDNA control as well as a DNase-treated, nonreverse transcribed tissue RNA sample to ensure that only the cDNA was quantified in each sample. The concentration of each target gene was based on the threshold cycle number (CT) for each sample, which was determined by the LightCycler® 96 Real-Time PCR System. The cDNA concentrations in each sample were determined according to the gene-specific standard curve. To normalize cDNA loading, all samples were run in parallel with the reference gene, β-actin, in the same plate. The relative gene expression of *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* were calculated as $F = (a/b)/(c/d)$, where a represents the target gene concentration from the treatment group, b represents the concentration of the internal gene from the treatment group, c represents the target gene concentration from the control group and where d represents the internal gene concentration from control group. Data were displayed as the means ± S.E.

2.6. Immersion challenge with the pathogenic strain S. agalactiae WC1535

Following administration probiotics for 15 days, twenty-five larvae from each replicate, including the C group, were bath exposed with strain *S. agalactiae* WC1535 to a final concentration of 1×10^6 cfu ml^{-1}. The WC1535 was provided by our Laboratory (Key Laboratory of Tropical & Subtropical Fishery Resource Application & Cultivation, Pearl River Fisheries Research Institute of CAFS) [8]. This strain was incubated at 37 °C for 48 h under anaerobic conditions in BHI media. The cultures were centrifuged (Beckman Coulter, AK, USA) at 2000 g for 5 min. The pellets were washed twice with phosphate-buffered saline (130 mM NaCl, 10 mM NaH_2PO_4, pH 7.2). The number of bacterial cells in each suspension was determine by turbidimetry. The water and pathogenic bacteria were changed completely every two days. Cumulative mortalities were recorded over a 20-day period.

2.7. Sampling of the gut for bacterial community profiling

For each treatment, gut samples from fifteen larvae were collected on the 15th day after probiotics administration, the 5th day after the cessation of probiotics administration (day 20) and one month after the cessation of probiotics administration (day 45). The intestines of all five fish were collected as one sample and three mixture samples were prepared for each group. All samples were frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.8. Gastrointestinal tract microbiome analysis

The DNA extraction and high-throughput sequencing are described in our previously published paper [8].

2.9. Statistical analysis

Results were expressed as the mean values ± S.E. (n = 3). Differences between treatments were determined using a one-way analysis of variance with the statistical software package SPSS Version 17.0. Differences were accepted as significant when $P < 0.05$. The Bray-Curtis dissimilarity was calculated based on square root-transformed relative abundance data. Principal coordinate analysis (PCoA) was performed to represent the samples in a low dimensional space; the relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Multivariate statistics were performed using the software package R version 3.5.1 (China). BLAST searches were used to identify the closest relative of selected OTUs within the genus *Lactococcus*.

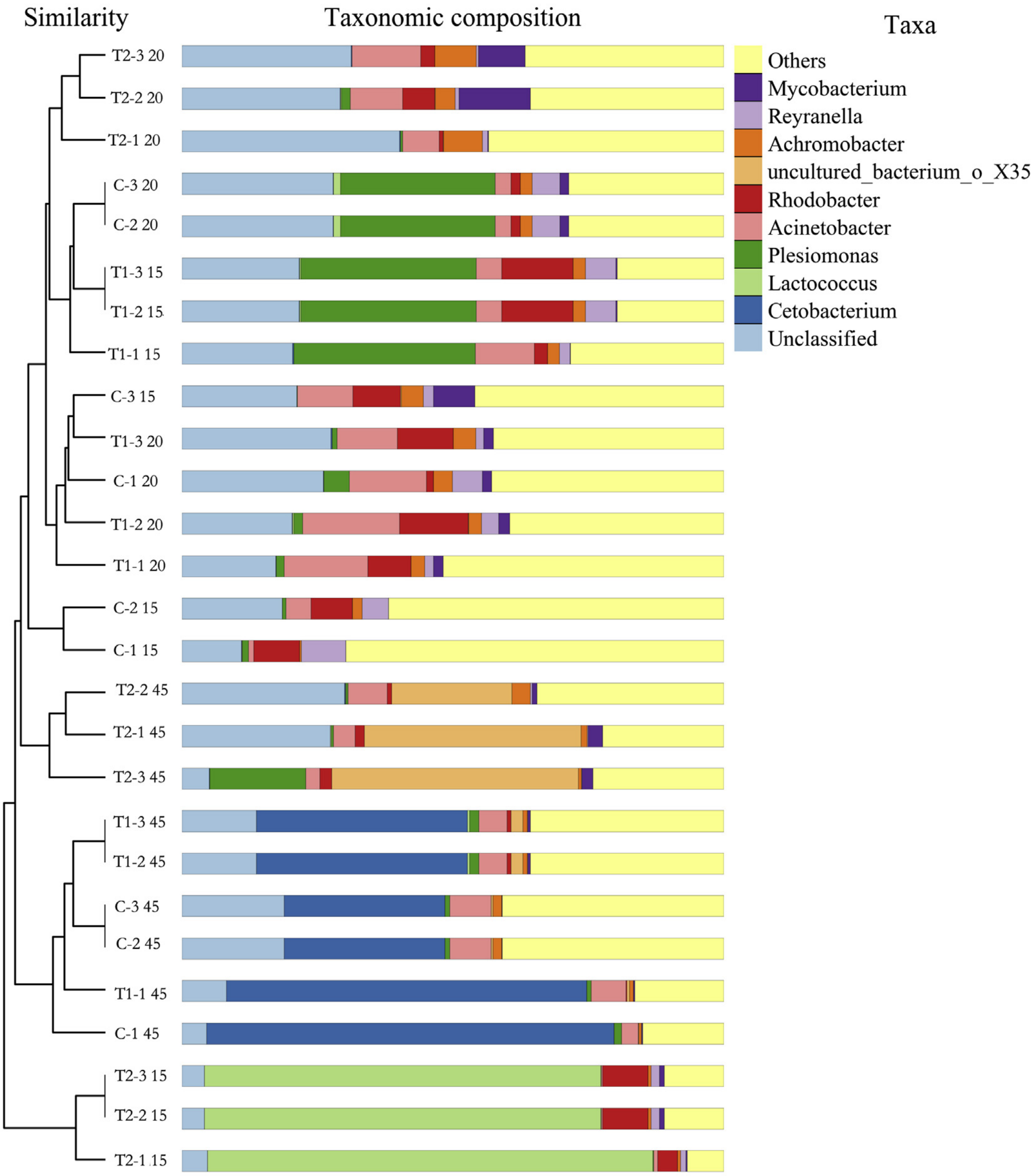

Fig. 5. Differences in intestinal bacterial communities at the genus level from larvae tilapia based on a Bray-Curtis distance matrix. C: control group; T1: JCM5805 was added to the water at a concentration of 1×10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration of 1×10^8 cfu ml^{-1}. 15: at day 15 of the experiment or after supplementation with JCM5805 for 15 days; 20: at day 20 of the experiment, which is 5 days after ceasing probiotic consumption; 45: at day 45 of the experiment, which is 1 month after ceasing probiotic consumption.

3. Results

3.1. Expression profiles of target genes after application of JCM5805

Within group T1, JCM5805 had no significant effect on *TLR7, TLR9, MyD88, IRF7* or *IFNα* gene expression ($P > 0.05$) except for *TLR9*, which was more highly expressed at day 10 after continuous administration of the probiotic ($P < 0.05$). However, these genes were more highly expressed in the T2 group; expression of *MyD88* and IRF7 was statistically higher on both the 5th and 10th days. *TLR9* and *IFNα* were also statistically higher in the T2 group than the C group ($P < 0.05$) on all of the three days (Fig. 2).

3.2. Expression profiles of target gene post-cessation of probiotics administration for 1 month

Following cessation of probiotics administration for 1 month, no significant difference was observed in the expression of *TLR7, TLR9, MyD88, IRF7* and *IFNα* genes in the intestine, liver and spleen between each group ($P > 0.05$) (Fig. 3).

3.3. Immersion challenge with pathogenic strain

The cumulative mortality of tilapia after the WC1535 challenge is shown in Fig. 4. Reduced mortality was observed in all treated groups compared with the control. However, only the T2 group showed significantly reduced mortality versus the control ($P < 0.05$).

3.4. Intestinal microbiological analysis

Following administration of probiotics for 15 days, the Shannon index of T2 was significantly lower than that of control larvae. However, the Simpson index of T2 was significantly increased (Table S1). After the cessation of probiotic administration, α-diversity indexes

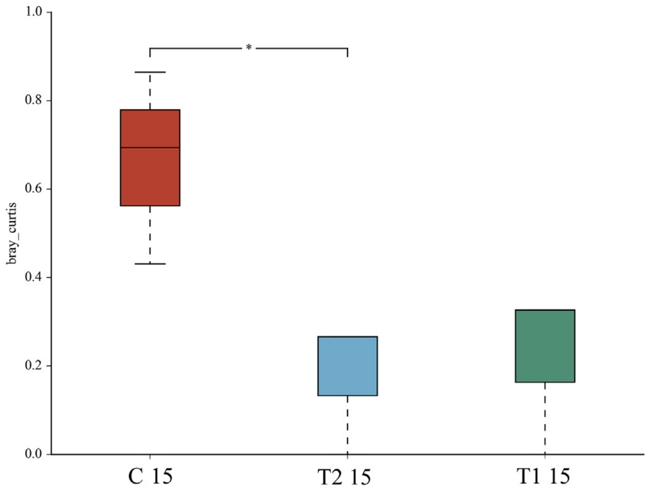

Fig. 6. Difference analysis of β-diversity between groups based on Bray-Curtis similarity. C: control group; T1: JCM5805 was added to the water at a concentration of 1×10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration of 1×10^8 cfu ml^{-1}. 15: at day 15 of the experiment or after supplementation with JCM5805 for 15 days. Asterisks indicate significant differences ($^*P < 0.05$).

among each group were not significantly different, with the exception of significantly lower Chao 1 in T1 and T2 after the cessation of probiotic administration for five days.

16S rRNA profiling of the gut microbiota from the T2 group on day 15 confirmed the presence of the probiotic strain in each sample at high relative abundances. At the end of the probiotic treatment (day 15), *Lactococcus* sp. accounted for approximately 76% of all bacteria in the gut (average relative abundance) of T2 (Fig. 5). The most abundant *Lactococcus* OTU (OTU1) had 100% sequence identity with the added probiotic strain of JCM5805 (Fig. S1). A comparison between the gut samples from the C and T2 treatments indicated a significant difference in the composition of the gut microbiota on the 15th day (Figs. 5 and 6). This difference reflected, in part, the high relative abundance of *L. lactis* in the gut of larvae from T2 and significantly reduced presence of these bacteria in the control, according to the SIMPER analysis results (contribution: 45.67%). Other discriminant OTUs were members of the genera *Acinetobacter*, *Reyranella* and *Achromobacter* (Table 2). The gut microbiota was not significantly different between the C and T1 groups on any of the sampling days (Fig. 5, Table 3). However, the replicate aquaria of the control treatments were more dispersed than those of the T1 group according to a cluster analysis of samples (Figs. 5 and 7).

At five days after the cessation in probiotic administration (day 20), the gut microbiota of C 20 and T1 20 was similar to the C 15 and T1 15 larvae (Fig. 7) and the relative abundance of these bacteria at day 20 was below the limit of detection. The most predominant OTUs in the T2 group were members of the genera *Acinetobacter*, *Rhodobacter*, *Achromobacter* and *Mycobacterium* (Fig. 5). These taxa were also among the most predominant bacteria observed in the gut of the C and T1 group on day 20, although they were present at different relative abundances. *Plesiomonas* was the most abundant (average 21%) member in C larvae but was below 1% in T2 larvae.

Table 3
Permutational MANOVA table testing for differences in the gut microbiota between the two treatments on each experimental day. Analysis was based on Bray-Curtis similarity of square root transformed relative abundance data. The permutation method was used on unrestricted permutations of raw data. A pseudo-F statistic was computed for each permutation and the Pr (perm) values give the proportion of permuted pseudo-F statistics that are equal to or greater than the original (unpermuted) pseudo-F statistic. SS and MS: Sum and mean of the squares, df: degrees of freedom, Pr: *P*-values calculated based on 999 Monte Carlo permutations drawn from the theoretical asymptotic permutation distribution.

PERMANOVA table for factor "Treatment" (C vs T1)						
Day 15						
Source	df	SS	MS	F-Model	R2	Pr (> F)
Management	1	0.29967	0.299666	3.0515	0.43274	0.1
Residuals	4	0.39282	0.098204		0.56726	
Total	5	0.69248			1.00000	
Day 20						
Source	df	SS	MS	F-Model	R2	Pr (> F)
Management	1	0.29237	0.292367	5.8239	0.59283	0.1
Residuals	4	0.20081	0.050201		0.40717	
Total	5	0.49317			1.00000	
Day 45						
Source	df	SS	MS	F-Model	R2	Pr (> F)
Management	1	0.13653	0.136531	1.8719	0.31879	0.2
Residuals	4	0.29175	0.072938		0.68121	
Total	5	0.42828			1.00000	

A comparison of the gut microbiota following the cessation of probiotic administration for one month (day 45) showed a clear difference in the composition of the gut microbiota between C and T2 groups (Fig. 7). On day 45, the probiotic strain remained below the detection level. The most predominant genera at day 45 in the T2 group were similar to those observed at day 20, albeit at a different relative abundance. However, the gut microbiota from the C and T1 groups were clearly clustered and separated from the samples on day 20 (PCoA). *Cetobacterium* was the most abundant genus in both treatments on day 45, with average relative abundances close to 50%.

Table 2
Similarity percentages (SIMPER) analysis of gut microbiota on day 15. Table indicates the foremost four characteristic OTUs from each group that contribute to the discrimination between the control and probiotic treatment (T2). Contribution values indicate the importance of each OTU (percentage) in increasing Bray-Curtis dissimilarity between the two treatments.

Similarity Percentages - species contributions					
Data type: Abundance					
Resemblance: Bray-Curtis dissimilarity					
Groups C 15 & T2 15					
Average dissimilarity = 83.34					
Genus (OTUs)	Group C Av. Abundance	Group T2 Av. Abundance	Av. Dissimilarity	Contrib%	Cum.%
Lactococcus (1)	0.03	76.15	38.06	45.67	45.67
Acinetobacter (14)	4.07	0.39	1.84	2.21	47.88
Reyranella (34)	4.69	1.4	1.65	1.98	49.86
Achromobacter (7)	3.09	0.55	1.27	1.52	51.38

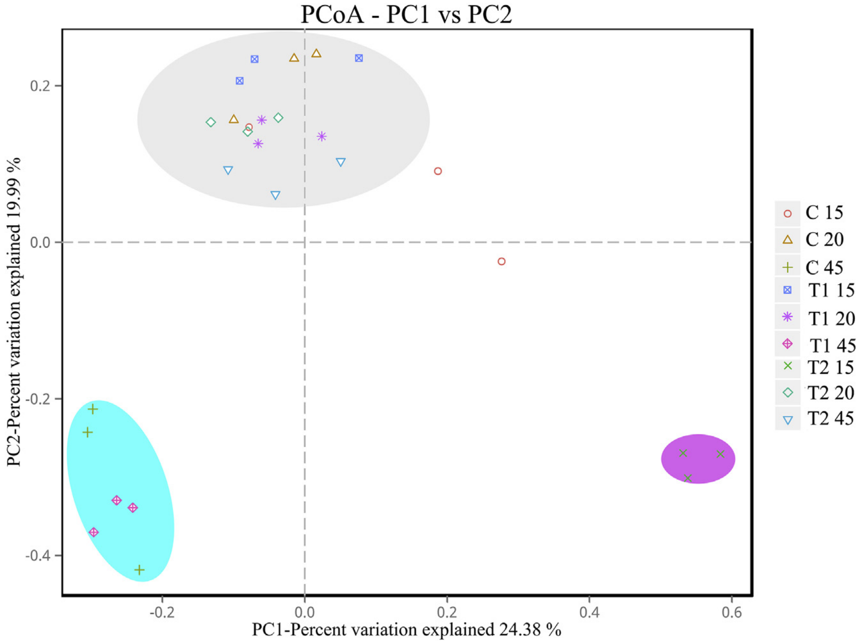

Fig. 7. Principal coordinate analysis (PCoA) of microbial communities in the gut and water samples of the control and probiotic treatments. The plots are based on the square root-transformed relative abundance data for the OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index.
C: control group; T1: JCM5805 was added to the water at a concentration of 1 × 10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration of 1 × 10^8 cfu ml^{-1}. 15: at day 15 of the experiment or after supplementation with JCM5805 for 15 days; 20: at day 20 of the experiment, which 5 days after ceasing probiotic consumption; 45: at day 45 of the experiment, which is 1 month after ceasing probiotic consumption.

3.5. Redundancy analysis

Redundancy analysis (RDA) was used to explore the correlations between bacteria at the phylum level using the target genes as variables in larvae intestinal tract samples (Fig. 8). RDA results showed that larvae from each treatment were grouped purposively on day 15 and 45. The T2 group and the high abundance of phyla in the intestinal tract were all distributed to the quadrants with high concentrations of target genes. RDA1 and RDA2 explained 41.31% and 17.36% of the total variance on the 15th day, respectively (Fig. 8A). The *TLR9*, *IRF7* and *IFNα* genes demonstrated strong and positive correlations with the abundance of Firmicutes, which were mainly limited to the genus *Lactococcus*. *MyD88* also demonstrated positive correlations with the abundance of Firmicutes. On day 45, the above correlations between genes and microbiota in each treatment were not obvious (Fig. 8B).

4. Discussion

The low survival and proliferation rate of probiotics in the fish gut limits their application. The embryonic development of fish occurs in natural waters and does not undergo the amniotic period like a mammal [2]. Application of probiotics during the embryonic development before the digestive tract has fully developed is more conductive to colonization of the probiotics in the intestine. In the research of Giatsis et al., the axenic larvae were used to enhance the colonization success of the probiotics [12]. However, the sterile samples were still contaminated during the test. In this research, the entire process was carried out under normal nonsterile conditions to better conform to aquaculture practices.

After administration of probiotics for 15 days, it is perhaps not surprising that the species diversity was significantly lower in this group, as indicated by significantly decreased Shannon index and increased Simpson of T2. This outcome is due to the absolute dominance of the administered probiotics JCM5805, which had a relative abundance of approximately 76%. The large distribution of JCM5805 demonstrated that it can be successfully transferred to the gut through the water. However, the gut microbiota was not significantly different between the control and low concentration group on any of the sampling days. This indicated that the effects of probiotics are dosage related [30] and higher dosages may enhance changes in the microbiota and/or immune responses [31]. As mentioned above, expression of almost all target genes and disease resistance of larvae in low concentration probiotic treatment were also not significantly different from those in the control group.

In the present study, JCM5805 was only transiently detected and thus was not included in the stable larval microbiota. Five days after cession of probiotic administration (day 20) in the larvae, the abundance of JCM5805 was already below the detection level. This finding demonstrates the challenge of successful colonization of probiotic strains in the fish gut. The presence of this strain in the gut can be expected only until a few days after probiotic discontinuation. This is consistent with previous studies which have reported that probiotic strains added through water or feed could be detected in the guts of fish and shrimp for only a few days after discontinuing application of the probiotic [12,32–34]. The persistence of probiotics in the gut is species-specific [35]. The dosage and duration of supplementation as well as the selection of probiotic strain/s might influence colonization success. Furthermore, the persistence of the probiotic might also depend on the developmental state of the animal [36–38]. In our previous study, JCM5805 was also only transiently detected in juvenile tilapia intestine after 6 weeks administration and was below the detection level after cession of probiotic administration for one week [8]. However, a main difference is that JCM5805 became the dominant species in larvae intestine in this research but did not become the dominant species in juvenile tilapia when probiotics were continuously administered. This difference may indicate that it is more feasible to inoculate the gut community with bacteria during early gut development [2].

The observed low persistence of the probiotic strain in the gut could indicate a lack of ecological preference or adaptability of the probiotic strain in the gut and/or host selectivity against the probiotic. Nevertheless, the gut communities remained different between treatments, even after discontinuation of the probiotic and despite receiving the same diet and same water source. The administration of JCM5805 at the high concentration significantly decreased the distribution of *Plesiomonas* in the gut 5 days after the cessation of probiotic administration (day 20). This is consistent with our previous study of JCM5805 in juvenile tilapia [8]. This genus was also found in the intestine of tilapia cultured in earthen ponds [39]. However, it was thought to be an opportunistic pathogen in aquaculture systems. Studies in gold fish [40] and grass carp [41] have confirmed that *Plesiomonas* was a pathogen. *Plesiomonas* has also been found to be an opportunistic pathogen in gibel carp that was more abundant in sick fish than in healthy fish [42].

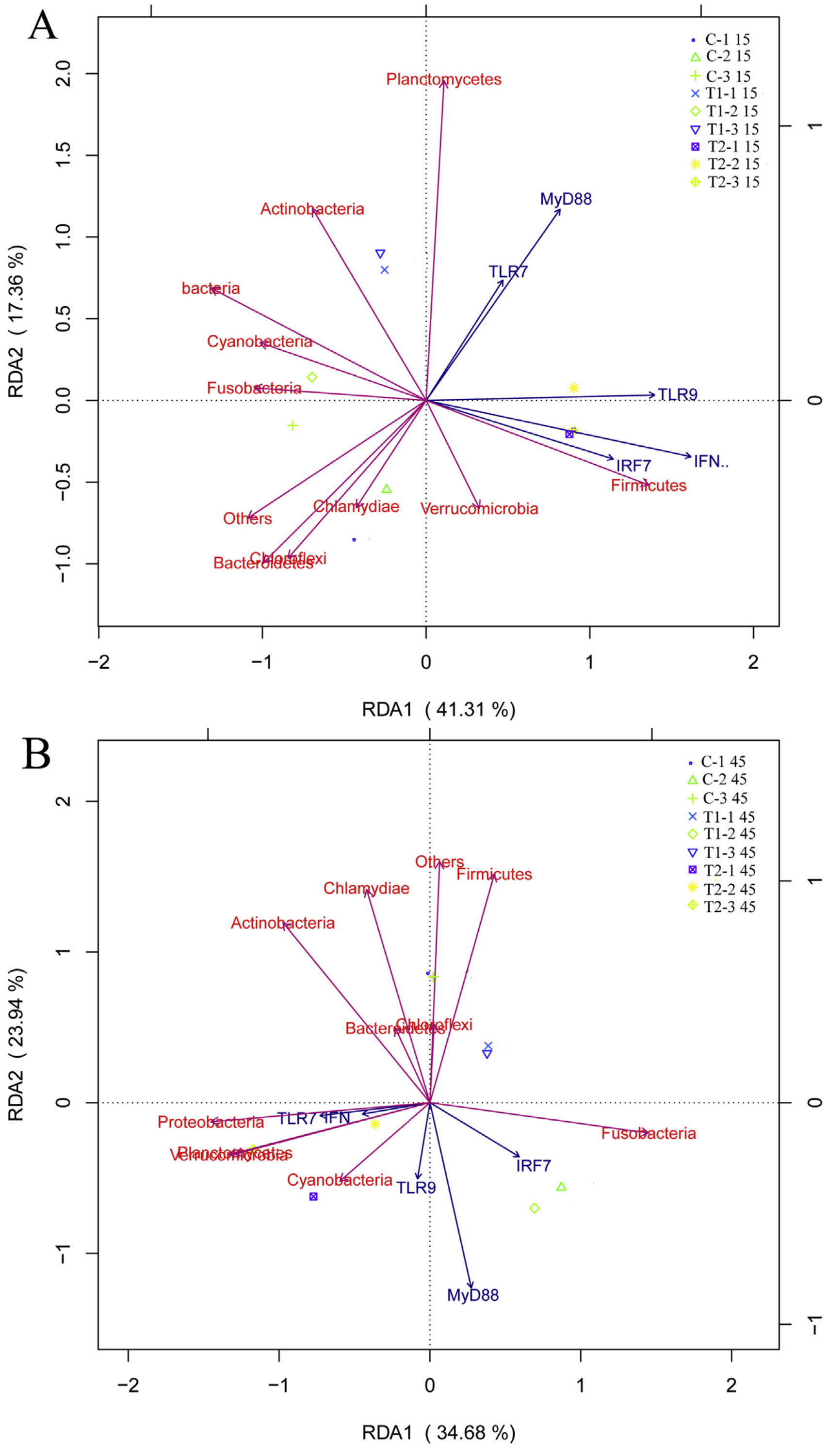

Fig. 8. Redundancy analysis (RDA) of phyla (red arrows) and target genes (blue arrows) in the intestinal tract samples of fish from the three groups. All taxa with abundances below 1% were grouped under others. RDA was performed with Canoco for Windows (V4.5). C: control group; T1: JCM5805 was added to the water at a concentration of 1×10^4 cfu ml^{-1}; T2: JCM5805 was added to the water at a concentration of 1×10^8 cfu ml^{-1}. 15: at day 15 of the experiment or after supplementation with JCM5805 for 15 days; 45: at day 45 of the experiment, which is 1 month after ceasing probiotic consumption. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The possibility to use probiotics or prebiotics to mitigate infection could remain if the richness of the host intestinal microbiota can be restored [42,43]. One month after the cessation of probiotic administration (day 45) the dominant bacteria in both the control and low concentration treatment groups was *Cetobacterium*. *Cetobacterium* has previously been isolated from the intestines of other fresh water fish species [44], including tilapia [35,45] and was found to ferment peptides and carbohydrates to produce vitamin B12 during the fermentation process [46]. However, *Acinetobacter* remained the dominant species in the high concentration group at day 45. After the discontinuation of probiotic administration, differences in the gut microbiota between treatments primarily reflected differences in the relative abundance of the genera *Cetobacterium*, *Plesiomonas*, *Acinetobacter*, *Rhodobacter*, *Achromobacter*, *Reyranella* and *Mycobacterium*. Most of these genera have been identified in previous studies on tilapia larvae and other fish species. The genus *Cetobacterium* has been reported in the guts of tilapia [8,35,45], carp [44,47] and *Siniperca chuatsi* [48]. The genus *Plesiomonas* has been reported in the guts of gold fish [40], grass carp [41], gibel carp [42], tilapia [8] and largemouth bass [49]. *Acinetobacter* has been observed in the guts of tilapia [8], Atlantic salmon [50], guppies [51] and gilthead sea bream [52]. Furthermore, members of the genus *Rhodobacter* have been reported in the guts of tilapia [8], grass carp [53] and gibel carp [42]. These findings identified a core microbiome in fish species and would infer that host-specificity for a particular microbial taxon is modulated by selective pressures within the host gut. These findings also indicate that these taxa are involved in major metabolic functions in the fish gut.

The gut microbiota alters the way the host reacts to infectious stimuli or particular bacterial taxa entering the gut [54]. Differences in the initial priming of the immune system in the probiotic group are potential mechanisms [12,55,56]. JCM5805 is a strain of lactic acid bacteria (LAB) that activates murine and human pDCs. pDCs express both TLR7 and TLR9 type receptors [26] which then induce the production of type I IFNs (IFNα) in a MyD88-dependent manner. Gerosa et al. reported that IFN-α produced from virus-stimulated pDC markedly increased the cytotoxic activity of NK cells [57]. IFNα produced from pDCs that were stimulated by JCM5805 might contribute largely to the activation of NK cells [24,25]. pDCs have been shown to be important not only as a producer of IFNs but also as a regulatory cell that controls the expression of various subsets of cytokines (such as the *IFN-α* here) [58]. JCM5805 could be taken up by the intestinal villus and contribute to the maintenance of gut homeostasis *in vivo* [24]. Administration of JCM5805 at a concentration 1×10^8 cfu ml^{-1} elevated the expression of target genes belonging to this signaling pathway in different levels and increased fish resistance to *S. agalactiae*. Preliminary analysis showed that the upregulation of related genes in this study was related to the presence of JCM5805 in the intestine. However, the abundance of a probiotic strain in the gut or feces is neither clear-cut proof of successful probiotic use nor evidence of probiosis. This point emphasizes the difficulty of establishing the precise relationships between health benefits and the presence and/or relative abundance of a specific nonpathogenic microbe [38]. Notably, inter-individual variation could certainly mask treatment effects by either type-I or type-II errors. Thus, more data points needed for higher statistical power should be included in future studies to verify whether the observed correlations are maintained.

In conclusion, JCM5805 was used in this research for the first time in tilapia at the early embryo development stage. After 15 days of treatment with 1×10^8 cfu ml^{-1}, JCM5805 was detected as the most abundant member in the fish gut. However, it did not successfully colonize and the presence of this strain in the gut can only be expected for a few days after probiotic discontinuation. The early microbial exposure of fish affects the gut microbiota at later life stages and led to the development of distinct communities of gut microbiota in tilapia larvae. The administration of JCM5805 at a concentration 1×10^8 cfu ml^{-1} boosted expression of *TLR7*, *TLR9*, *MyD88*, *IRF7* and *IFNα* genes, and improved disease resistance to *S. agalactiae* in Nile tilapia. Further studies on the effects of probiotics on body immunity should provide a better understanding of the correlation between immunity and gut microbiota of fish.

Acknowledgements

This work was supported by the China Agriculture Research System (CARS-46), Natural Science Foundation of Guangdong Province (2016A030313146) and Special Scientific Research Funds for Central Non-profit Institutes, Chinese Academy of Fishery Science (grant No. 2016HY-ZD1403).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.022.

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