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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Oral delivery of *Bacillus subtilis* spores expressing *Clonorchis sinensis* paramyosin protects *grass carp* from cercaria infection

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Abstract

Clonorchis sinensis (*C. sinensis*), an important fishborne zoonotic parasite threatening public health, is of major socio-economic importance in epidemic areas. Effective strategies are still urgently expected to prevent against *C. sinensis* infection. In the present study, paramyosin of *C. sinensis* (CsPmy) was stably and abundantly expressed on the surface of *Bacillus subtilis* spores. The recombinant spores (B.s-CotC-CsPmy) were incorporated in the basal pellets diet in three different dosages (1×10^5, 1×10^8, 1×10^{11} CFU/g pellets) and orally administrated to grass carp (*Ctenopharyngodon idella*). The immune responses and intestinal microbiota in the treated grass carp were investigated. Results showed that specific anti-CsPmy IgM levels in sera, skin mucus, bile, and intestinal mucus, as well as mRNA levels of IgM and IgZ in the spleen and head kidney, were significantly increased in B.s-CotC-CsPmy-10^{11} group. Besides, transcripts levels of IL-8 and TNF-αin the spleen and head kidney were also significantly elevated than the control groups. Moreover, mRNA levels of tight junction proteins in the intestines of B.s-CotC-CsPmy-10^{11} group increased. Potential pathogenetic bacteria with lower abundance and higher abundances of candidate probiotics and bacteria associated with digestion in 1×10^{11} CFU/g B.s-CotC-CsPmy spores administrated fishes could be detected compared with control group. The amount of metacercaria in per gram fish flesh was statistically decreased in 1×10^{11} CFU/g B.s-CotC-CsPmy spores orally immunized group. Our work demonstrated that *B. subtilis* spores presenting CsPmy on the surface could be a promising effective, safe, and needle-free candidate vaccine against *C. sinensis* infection for grass carp.

Keywords *Clonorchis sinensis* · *Bacillus subtilis* spore · Paramyosin · Oral vaccine · Intestinal microbiota · Grass carp

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Introduction

Clonorchis sinensis (*C. sinensis*), an important fishborne zoonotic trematodes parasite, is prevalent in Asian countries and regions including China, South Korea, northern Vietnam, and Russia. Adult worms of *C. sinensis* live in the intrahepatic bile duct of definitive host. In addition to human beings, various other kinds of mammals can be definitive hosts of *C. sinensis*, such as cat, dog, pig, rabbits, etc. As reported, the average prevalences of *C. sinensis* infection in dogs and cats were 20.5% and 41.8% in the Pearl River Delta region which is the most important endemic area in Guangdong province (Lin et al., 2011). And nearly 35 million people are estimated to be infected with *C. sinensis* globally, of whom approximately 15 million are in China (Lai et al. 2016; Qian et al. 2016; Tang et al. 2016b) and bring a series of diseases like indigestion, biliary inflammation, bile duct obstruction, even liver cirrhosis, and hepatic carcinoma (Tang et al. 2016b). Accumulating evidence demonstrated that there is an aetiological relation between clonorchiasis and cholangiocarcinoma in human beings (Lun et al. 2005; Machicado and Marcos 2016; Zheng et al. 2017). However, we still lack effective strategy to completely prevent the spread of *C. sinensis* at present (Tang et al. 2016b). Human beings or other definitive hosts get infected by ingesting raw or undercooked fishes (the second intermediate hosts) containing live metacercaria (Lun et al. 2005). On the one hand, in epidemic areas wild animals served as the definitive hosts (reservoir hosts) for *C. sinensis* (Qian et al. 2016) which could be infectious source. For example, in Southern China, a large number of dogs and cats roam freely in rural settings, and the presence of these animals in proximity with people may represent a risk of parasitic zoonoses including *C. sinensis* (Fang et al. 2015; Nguyen et al. 2018). On the other hand, eating raw fish has been deeply rooted in culture of the area. In previous, most vaccine trials focused on the definitive host of *C. sinensis* instead of the intermediate hosts including freshwater fishes or snails (the first intermediate hosts). Protein-based or nucleic acid-based vaccine trials have been conducted on the rat model, but none of the vaccine candidates brought a protective effection (worm reduction rate) of more than 70%(Qian et al. 2016; Tang et al. 2016b). Freshwater fishes (e.g., *Ctenopharyngodon idellus*, *Carassius auratus*, and *Hypophthalmichthys nobilis*) serve as the second intermediate host for *C. sinensis*. Hence, we speculate that cutting off the life cycle of *C. sinensis* by preventing the cercaria invasion or metacercariae formation in freshwater fish might be an efficacious tactic to control the prevalence of *C. sinensis*.

Vaccine has been the most effective method for combating infectious disease in aquaculture industry (Gudding and Van Muiswinkel 2013; Plant and Lapatra 2011). Compared with other immunization routes (injection and immersion route), oral vaccine is a preferable route as it is needle-free, no size limitation, lower cost, and more convenient for farmer operation (Plant and Lapatra 2011). However, oral immunization suffers from antigen degradation in the gastrointestinal tract of fish, which will affect the protective effect (Quentel and Vigneulle 1997). A considerable amount of investigations have proved that spores of *Bacillus subtilis* (*B. subtilis*) were a potent antigen delivery platform for oral vaccine (Ricca et al. 2014; Rosales-Mendoza and Angulo 2015; Tavares Batista et al. 2014). Because *B. subtilis* spores can survive extreme environment in the gastrointestinal tract, thus protect the antigens from digestion and degradation (Duc le et al. 2003). Besides, *B. subtilis* were widely employed as probiotic additives as it enhances the growth performance, digestive enzyme activities, immune responses, and disease resistance of fishes or shrimps (Liu et al. 2017; Sanchez-Ortiz et al. 2016; Truong Thy et al. 2017; Wang et al. 2010). *B. subtilis* spores were widely investigated as a delivery vehicle for the oral vaccine in aquaculture industry (Fu et al. 2010; Valdez et al. 2014).

In our previous work, an oral delivery system based on *B. subtilis* spore has been successfully established and confirmed to be valid and feasible (Jiang et al. 2017; Tang et al. 2017; Zhou et al. 2008). Enolase and cysteine protease of *C. sinensis* (CsENO and CsCP) were expressed on the surface of *B. subtilis* spore, and the recombinant spores elicited both humoral and mucosal immune response in grass carp by oral immunization (Jiang et al. 2017; Tang et al. 2017). But the protect effect against *C.sinensis* and the safety of spores need further study.

Paramyosin (Pmy), an invertebrate muscle-associated multifunctional protein, has emerged as a promising vaccine candidate for various kinds of parasites (e.g., *Schistosoma mansoni*, *Schistosoma japonicum, Taenia solium, Fasciola gigantica*, etc.) (Abou-Elhakam et al. 2013; Jiz et al. 2015; Vazquez-Talavera et al. 2001). Paramyosin of *C. sinensis* (CsPmy, Accession number: JQ041818.1) was found to be highly expressed at the stage of adult worm, metacercariae, and cercaria. Both prokaryotic expressed protein and DNA vaccine of CsPmy brought encouraging protect effect in rat models. Furthermore, CsPmy was confirmed to be an important component of cyst wall of metacercariae, which suggested us that CsPmy may play a vital role in metacercariae formation in freshwater fishes (Wang et al. 2012).

In the present study, we aimed to explore whether oral administration with *B. subtilis* spores expressing CsPmy on the surface would be an effective and safe measure to protect grass carp from *C. sinensis* infection. CsPmy were fusion expressed on the surface of *B. subtilis* spores with CotC, a coat proteins of *B. subtilis* spores, and the immune response and protect effect in grass carp elicited by oral administration with the recombinant spores were evaluated. Besides, its influence on intestines and the intestinal microbiota of immunized grass carp were also investigated by using qRT-PCR and MiSeq high-throughput sequencing, respectively.

Method and materials

Fishes and recombinant *B. subtilis* spores

Healthy grass carp weighing 20–25 g were acquired from Seedling Production Base of Pearl River Fisheries Institute (Guangzhou, China) and kept in the laboratory for acclimation for 2 weeks. Subsequently, fishes were divided into several tanks with the same volume of water (35 fishes per tank) and fed daily. Before experiments, fishes were randomly sampled for metacercaria detection according to the methods described previously (Liang et al. 2009) to confirm negative infection.

B. subtilis spore fusion expressing CotC-CsPmy (B.s-CotC-CsPmy) and *B. subtilis* spore expressing CotC (B.s-CotC) as the control were obtained by our previous study and preserved in our lab (Sun et al. 2018).

Extraction of coat protein of spores

B. subtilis WB600 strains with B.s-CotC-CsPmy or B.s-CotC was cultured in Difco Sporulation Medium (DSM, BD, Franklin Lakes, USA) for 24 h as described. Spores were harvested and washed with 1 M NaCl, 1 M KCl and distilled water in turn (Nicholson WL 1990). Finally, spores were re-suspended in distilled water and treated in 68 °C for 1 h to kill residue vegetative cell. They were counted and stored in − 80 °C prior to use.

To extract the coat proteins of spores, spores were resuspended with sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) extraction buffer (0.5% SDS, 0.1 M DTT, 0.1 M NaCl) and incubated at 37 °C for 2 h (Tang et al. 2016a). Followed by six times wash with 1 M Tris-HCl buffer (pH 8.0), the spores were suspended in 5 ml broken buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1 mM PMSF) and ultrasonicated for 5 min. After centrifugation, the coat proteins were collected from the sediment, and the supernatant was preserved for further analysis as well (Tang et al. 2016a; Zhou et al. 2008).

Protein identification by mass spectrometry

B.s-CotC-CsPmy spores were analyzed by 12% SDS-PAGE. The corresponding expression band of CotC-CsPmy in the polyacrylamide gel was digested with trypsin as described before (Katayama et al. 2001). The peptides were analyzed through liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) with an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Finnigan, USA). Mascot V2.3 search engine (Matrix Science, London, UK) was applied for protein identification using the following search parameters: *Clonorchis sinensis* database; two missed cleavage site; fixed modifications of Carbamidomethyl (C); partial modifications of Acetyl (Protein N-term), Deamidated (NQ), Deoxidation (W), Oxidation (M); and ± 30 ppm for precursor ion tolerance and ± 0.15 Da for fragment ion tolerance.

Diet preparation and oral administration

The basal diet was a commercial pellet (Taifeng, Foshan, China, with the chemical composition of crude protein ≥ 30.0%, crude fat ≥ 3.0%, ash ≤ 13.0%, crude cellulose ≤ 12.0%, lysine ≥ 1.3%, and total phosphorus ≥ 0.7%). Five experimental diets were prepared in accordance with the methods described before (Jiang et al. 2017; Tang et al. 2017). Naïve group was treated with basal feed without spores. The control group (B.s-CotC-10^8 group) was administrated with basal feed plus B.s-CotC spores (1×10^8 CFU/g). Experimental groups were managed with 1×10^5 CFU/g, 1×10^8 CFU/g, or 1×10^{11} CFU/g of B.s-CotC-CsPmy spores. To avoid dispersion of the spores in water, spores and basal feed were coated with an equal volume of cod liver oil (Tang et al. 2017). The diets were dried and stored at − 20 °C until use. For oral immunization, fishes in each group were hand-fed with 2% of their initial body weight twice per day (at 09:00 am and 17:00 pm) for 6 weeks. Thereafter, fishes were fed with basal diet till the end of the experiment.

Samples collection

Five fishes were randomly sampled from each group at week 2, 4, and 6 after the beginning of the immunization. Grass carp was euthanized with overdose of eugenol mixture, and then skin mucus, blood, gallbladder, intestinal mucus, head kidney, and spleen were collected as described method (Guo et al. 2016). Briefly, skin mucus was gently scraped with a glass slide, then diluted with 0.5 ml sterile PBS, centrifuge at 4 °C, 4500 rpm for 20 min, and stored in −80 °C (Tang et al. 2017). Blood was collected from the caudal vein using a 1 ml injector, clotted in the room temperature for 2 h, and then sera were separated by centrifugation (4 °C, 4000 rpm for 15 min) and stored in − 20 °C until use (Jiang et al. 2017). The intestine was aseptically isolated, lavaged with 0.5 ml sterile PBS for several times, and centrifugated (4 °C, 4500 rpm for 20 min). The supernatant of the lavage fluid was stored at − 20 °C. The spleen and head kidney of each fish was dissected and preserved in sample protector (Takara Bio, Otsu, Japan) in − 80 °C. About 8 weeks after the beginning of administration, another three fishes in each group were sampled and euthanized, and the whole intestines of the three fishes were aseptically excised for gut microbiota analysis.

Analysis of CsPmy-specific IgM

Indirect enzyme-linked immunosorbent assay (ELISA) was employed to analyze the specific antibody levels against CsPmy in samples including skin mucus, sera, bile, and

intestinal mucus. In brief, the 96-well microtiter plates were coated with 100 ml per well of 5 μg/ml rCsPmy carbonate-bicarbonate buffer (0.05 M, pH 9.6) in 4 °C overnight. After washing with PBST three times, the plates were blocked with blocking buffer containing 5% skimmed milk for 2 h at 37 °C. Meanwhile, sera, bile, skin mucus, and intestinal mucus was diluted with 1% BSA in PBST at a dilution of 1:100, 1:100, 1:20, 1:20, respectively. Then 0.1 ml of diluted samples were added to each well as primary antibody and incubated for 2 h at 37 °C. After three times washing, 0.1 ml of HRP-conjugated rabbit anti-grass carp IgM (diluted at 1:7000) was added and incubated at 37 °C for 1 h. The plates were washed for 3 times again, 0.1 ml of tetramethylbenzidine (TMB, BD, USA) was added and reacted at RT for 10 min. Finally, the reaction was terminated by adding 50 μl of 2 M H_2SO_4, and the optical density value at 450 nm was detected by a microplate reader.

mRNA levels of immune-related molecules and tight junction proteins by quantitative real-time polymerase chain reaction (qRT-PCR)

About 6 weeks after the beginning of oral administration, total RNA was extracted from the head kidney, spleen, foregut, midgut, or hindgut tissues of grass carp with TRIzol reagent (TransGen Biotech, Beijing, China) and was reverse transcribed into the first strand of cDNA by using an All-in-One First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). mRNA levels of immunoglobulin M (IgM), immunoglobulin Z (IgZ), tumor necrosis factor α (TNF-α), and interleukin 8(IL-8) in the head kidney and spleen were analyzed by qRT-PCR. Additionally, transcription levels of gene encoding tight junction proteins including ZO-1, occludin, claudin b, and claudin c were also detected. β-actin was used as an internal reference gene (Feng et al. 2015). The specific primers were designed according to the published grass carp sequences and were listed in Table S1. The qRT-PCR procedure was carried out by the process described (Jiang et al. 2017). Data were analyzed by the $2^{-\Delta\Delta Ct}$ method with CFX Manager Software.

Gut microbiome analysis

The whole intestines of the three fishes were aseptically excised and opened and rinsed with sterilized PBS to remove the contents. Intestines from three fishes in the same group were collected into one sterile centrifuge tube. The intestinal samples were homogenized with sterilized PBS. The genomic DNA was extracted using OMEGA D3350 Bacterial DNA Kit (Omega, USA) according to manufacturer's protocols. The V3-V4 regions of 16S ribosomal DNA of bacteria were amplified, purified, and pooled in equimolar and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform (OE Biotech, Shanghai, China).

Data was demultiplexed, quality filtered, and analyzed with QIIME(v1.8.0) and UCHIME (v4.2)(Caporaso et al. 2010; Edgar et al. 2011). Operational taxonomic units (OTUs) were generated using VSEARCH (v2.4.2) software with 97% similarity cutoff. All representative reads were annotated and blasted against Silva database (v123) using RDP classifier (v2.2) with the confidence threshold of 70% (Hao et al. 2017a). Bias-corrected Chao1 richness estimator (Chao 1) was applied to evaluate the community richness. Shannon-Wiener Index (Shannon) and Simpson's diversity index (Simpson) was used for diversity evaluation for each sample. The structures of the microbial community in different samples were compared based on the column diagram, heat map analysis, and principal-component analysis (PCA) (Hao et al. 2017a). The bacterial abundance was analyzed among the groups. All the Illumina sequencing data has been deposited in the NCBI Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra/), and the accession numbers were SRR10244385/ SRR10244384/ SRR10244383/SRR10244382/SRR10244381.

Challenge infection

Parafossarulus striatulus, the first intermediate host of *C. sinensis*, were captured from Yangshan county, Guangdong province, China, and were elaborately cultivated in our laboratory. After 2 weeks acclimation, the *Parafossarulus striatulus* were fed with *C. sinensis* eggs. Ninety days later, snails were checked every day for cercariae release (Liang et al. 2009). Water with living cercariae was collected every day and was equally divided into each tank to infect fishes in the naïve group ($n = 5$), B.s-CotC-10^8 group ($n = 5$), and B.s-CotC-CsPmy-10^{11} group ($n = 5$). Challenge infection lasted for 7 days. Four weeks post the challenge infection, all fishes were sacrificed. The flesh was weighted, cut into pieces, and separately digested with artificial gastric juice for *C.sinensis* metacercaria detection. The number of metacercaria in per gram flesh of fish was calculated.

Statistical analysis

Data in experiments were expressed as the mean ± SD values. Student's *t test* was applied to analyze statistical differences in mRNA levels and number of metacercaria in per gram flesh among different groups using GraphPad Prism 5 software (version 5.0 for windows). One-way analysis of variance (ANOVA) with Tukey tests were used to analyze statistical differences of antibody levels with GraphPad Prism 5 software (version 5.0 for windows). The difference was considered as statistically significant if the P value < 0.05.

Results

Acquirement of recombinant spore of B.s-CotC-CsPmy

SDS-PAGE and western blotting by using anti-CsPmy antibody showed that CotC-CsPmy expressed in coat proteins extracted from the recombinant *B. subtilis* spores (Fig. 1A, B). Flow cytometry showed that 58.4% of B.s-CotC-CsPmy spores exhibited a strong fluorescence intensity ranging from 10^4 to 10^6 by anti-CsPmy serum as a primary antibody (Fig. 1C). LC-MS/MS confirmed the protein band with 100 kDa as CsPmy (Fig. 1D and Table S2).

Specific IgM levels in sera, skin mucus, intestinal mucus, and bile

Compared with Naïve groups and B.s-CotC groups, specific anti-CsPmy IgM levels in sera, skin mucus, intestinal mucus, and bile samples from fishes of B.s-CotC-CsPmy groups raised since week 2 and went on rising till week 6 (Fig. 2). In the skin mucus, IgM level significantly elevated from week 4 (Fig. 2B), which raised later than those in sera, intestinal mucus, and bile samples (Fig. 2A, C, D). Compare with the 10^5 dosage group, 10^8 and 10^{11} dosage groups elicited higher IgM level. The highest one was 10^{11} dosage group (Fig. 2).

mRNA levels of IgM and IgZ in the head kidney and spleen

Compared with naïve groups, the mRNA levels of IgM and IgZ in middle dosage (1×10^8 CFU/g B.s-CotC-CsPmy spores) and high dosage (1×10^{11} CFU/g B.s-CotC - CsPmy spores) were significantly increased both in the head kidney and spleen. The mRNA levels of IgM and IgZ in 1×10^{11} CFU/g groups were the highest. There was no significant difference in mRNA levels of IgM and IgZ in the head kidney and spleen between naïve group and B.s-CotC group. Compared with naïve group, there was no statistical increase of mRNA levels of IgM and IgZ in 1×10^5 CFU/g group except IgZ mRNA level in the head kidney (Fig. 3).

Proinflammatory cytokines in the head kidney and spleen

In the head kidney, TNF-α mRNA level of 1×10^{11} CFU/g B.s-CotC-CsPmy group were significantly higher than those in naïve group and B.s-CotC group (Fig. S1A), while no significant differences were detected between those in 1×10^5 and 1×10^8 CFU/g B.s-CotC-CsPmy groups. IL-8 mRNA level was significantly raised compared with both naïve group and B.s-CotC group. In spleen, mRNA levels of TNF-αand

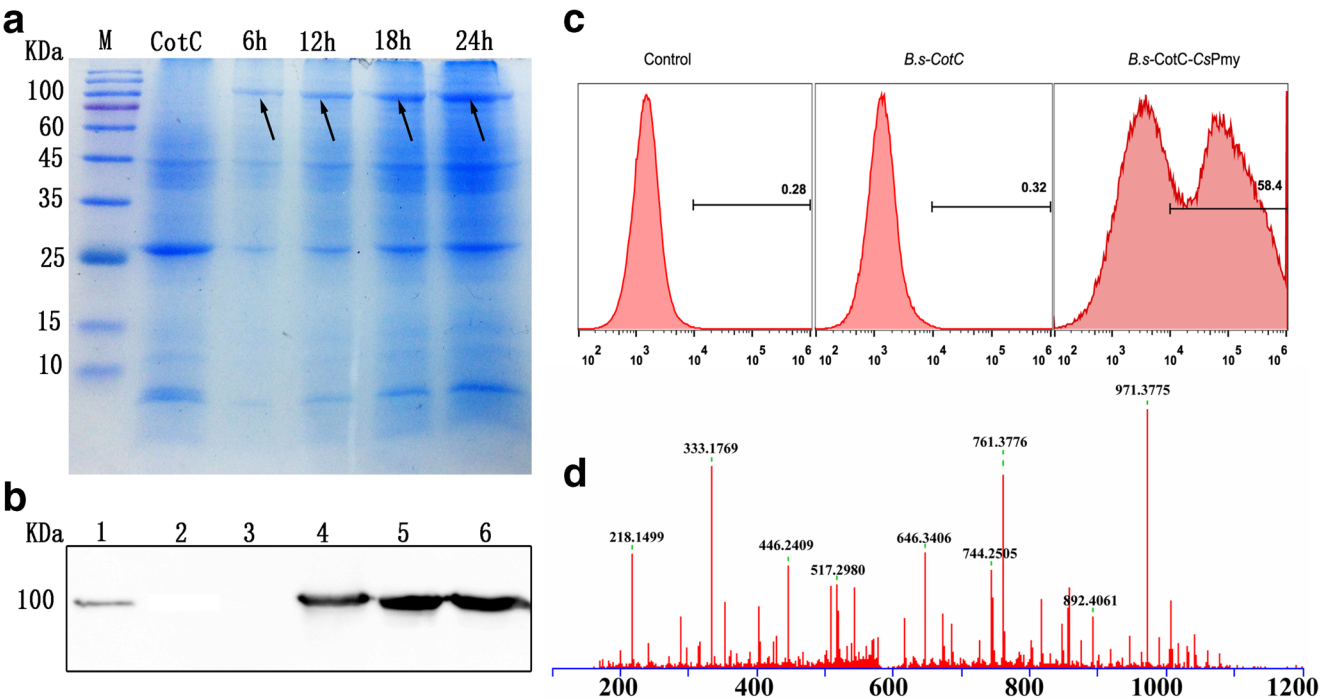

Fig. 1 Analysis of CsPmy expression on spore surface. A 12% SDS-PAGE analysis of CsPmy expression on *B. subtilis* spore at the different time point. The dark arrows indicated the band of CsPmy. M, protein marker; CotC, B.s-CotC spores; 6 h, 12 h, 18 h, 24 h, sporulation induction time of B.s-CotC-CsPmy. **B** Western blotting of CsPmy expression in coat proteins of B.s-CotC-CsPmy spores. Lane 1, recombinant protein of CsPmy; lane 2, coat protein of B.s-CotC spore; lane 3, propagules of B.s-CotC-CsPmy; lane 4–6, coat protein of B.s-CotC-CsPmy spore-induced for 6 h, 12 h, and 24 h, respectively. **C** Flow cytometry; Control, *B. subtilis* spores containing no plasmid; B.s-CotC, *B.subtilis* spore containing PEB03-CotC plasmid; B.s-CotC-CsPmy, *B. subtilis* spore harboring PEB03-CotC-CsPmy plasmid. **D** LC-MS/MS analysis of coat proteins of B.s-CotC-CsPmy. The paramyosin from *C. sinensis* was identified in coat proteins

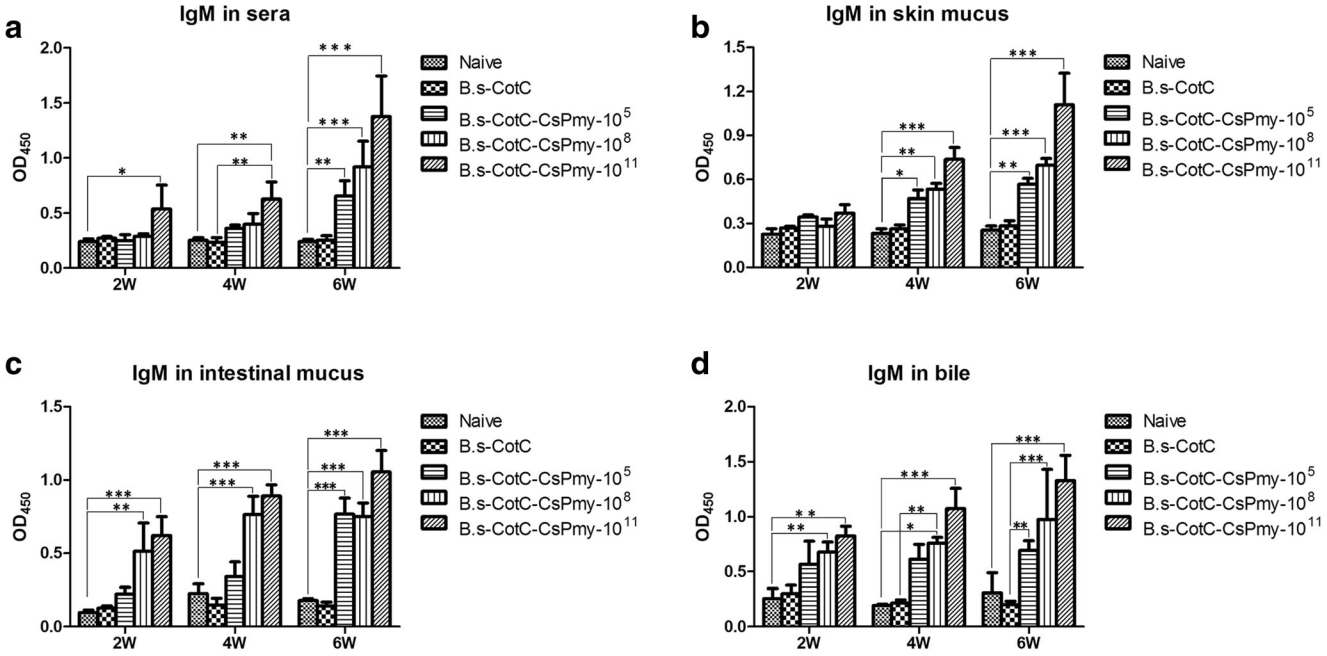

Fig. 2 Specific anti-CsPmy IgM levels in oral immunized grass carp Grass carp was orally administrated with different dosage of recombinant spores. Specific anti-CsPmy antibodies were determined by ELISA in week 2, 4, and 6 after the beginning of the oral immunization. (**A**) sera, (**B**) skin mucus, (**C**) intestinal mucus, (**D**) bile. Naïve, basal diet group; B.s-CotC-10^8: basal diet plus 1×10^8 CFU/g B.s-CotC spores. B.s-CotC-CsPmy-10^5, 10^8, 10^{11}, basal diet plus 10^5, 10^8, 10^{11} CFU/g B.s-CotC-CsPmy spores, respectively. Data were represented as mean ± SD. Statistical significance was analyzed by the Dunnett's test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

IL-8 significantly increased in 1×10^8 and 1×10^{11} CFU/g B.s-CotC-CsPmy groups compared with those in naïve group (Fig. S1B). No differences were detected between B.s-CotC and naïve group (Fig. S1).

Transcriptional levels of genes encoding tight junction proteins

To investigate the influences of oral administration of spores on the integrality of intestines mucosa, transcriptional levels of genes encoding tight junction proteins (ZO-1, occludin, claudin b, claudin c) in foregut, midgut, and hindgut of grass carp were analyzed. In the foregut, transcriptional levels of ZO-1, occludin, claudin b, and claudin c were the highest in fishes fed with 1×10^{11} CFU/g B.s-CotC-CsPmy spores, which were significantly increased compared with those in fishes from naïve or B.s-CotC groups (Fig. S2A). The ZO-1 mRNA level in the midgut and hindgut were not dominantly affected by spores administration (Fig. S2B-a, C-a). Transcriptional levels of occludin, claudin b, claudin c in midgut, and hindgut of grass carp from 1×10^{11} B.s-CotC-CsPmy spores group were obviously elevated compared with those of other groups (Fig. S2B, C).

Intestinal microbiome analysis

In total, 963 OTUs were generated from the samples through Illumina MiSeq sequencing, and these OTUs belong to 11 different phyla. Good's nonparametric coverage estimator (good's coverage) of each sample tend to approach 1 (Fig. 4A-a). Chao 1 index was used to estimate microbial community richness of the intestinal microbiota. The Chao 1 value in 1×10^{11} CFU/g B.s-CotC-CsPmy spores (BH) group was the highest compared with those of other groups (Fig. 4A-b). Shannon index and Simpson index were calculated to evaluate microbial community diversity. Shannon and Simpson index value of BH group was the highest among the groups. Diversity index of B.s-CotC spores fed group (BC), 1×10^5/ 10^8/ 10^{11} CFU/g B.s-CotC-CsPmy spores treated groups (BL, BM, BH) were higher than that of control group (NC) (Fig. 4A-c, d). The shared and unique OUTs among the samples were investigated and showed in the Venn diagram. A total of 84 OTUs were shared by all the 5 groups, and 45 unique OUTs were detected in BH group, which was the largest number among groups (Fig. 4B).

Principal components analysis (PCA) with weighted UniFrac method was applied to analyze the relationships between bacterial communities from different groups. The score plot of PCA revealed that microbial communities from BC, BL, BM, and NC groups were gathered at left-hand side of the plot along the second principal component axis (PC2), which accounts for 72.1% of the total variations. However, BH group was on right side of the graph along PC2. BL group was separated from the other groups along PC1, which represented 14.08% of the total variations. In general, the two axes (PC1 and PC2) explained 86.18% (Fig. 4C). In addition, the

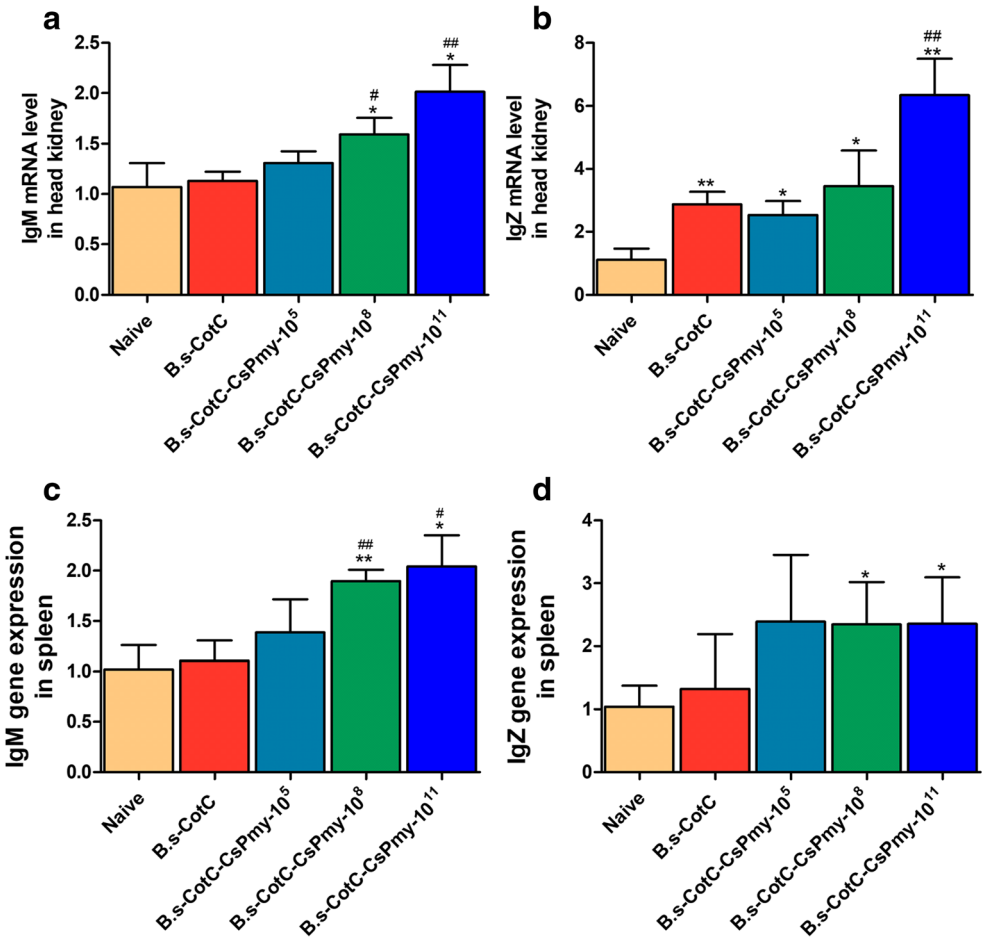

Fig. 3 The relative mRNA levels of IgM and IgZ in the head kidney and spleen. The mRNA levels of IgM and IgZ were analyzed by qRT-PCR at 6 weeks after the beginning of the immunization. (**A**) IgM mRNA level in the head kidney; (**B**) IgZ mRNA level in the head kidney; (**C**) IgM mRNA level in the spleen; (**D**) IgZ mRNA level in the spleen. Naïve, basal diet group; B.s-CotC-10^8: basal diet plus 1×10^8 CFU/g B.s-CotC spores. B.s-CotC-CsPmy-10^5, 10^8, 10^{11}, basal diet plus 10^5, 10^8, 10^{11} CFU/g B.s-CotC-CsPmy spores, respectively. $^*P < 0.05$, $^{**}P < 0.01$ (compared with naïve group); $\#P < 0.05$, $\#\#P < 0.01$ (compared with B.s-CotC-10^8 group)

differences among the groups were also observed in the heatmap, which showed the bacteria ranking top 30 in the abundance. (Fig. 4D).

Proteobacteria, Fusobacteria, Firmicutes, and *Spirochaetae* were main bacteria phyla in all grass carp, accounting for more than 99% of bacteria. *Firmicutes* were the most predominant phylum in BH group (25.38%), followed by *Fusobacteria* (8.2%). In NC, BC, and BM groups, *Proteobacteria* had the highest abundance accounted for 23.74%, 58.55%, and 51.15%, respectively (Table 1).

The abundance of potential pathogenetic bacteria such as *Pseudomonas* and *Flavobacterium* were detected in samples. The amount of sequences related to *Pseudomonas* in BH group decreased compared with that in NC group. *Flavobacterium* with lower abundance was found in NC and BL groups but not in BH, BC, and BM groups. (Table 2). Candidate probiotics including *Lactobacillus*, *Streptococcus,* and *Micrococcus* were also detected and analyzed. Higher-abundance *Lactobacillus* and *Streptococcus* genus were found in BH group compared with NC group. *Micrococcus* could not be detected in all samples (Table 2).

In addition, sequences related to bacteria associated with digestion were also investigated. The abundance of *Rikenellaceae, Ruminococcaceae, and Lachnospiraceae* (at family level) varied in different groups, but they were dramatically increased in BH group compared with other groups (Table 3). The abundances of *Odoribacter*, *Desulfovibrio*, and *Alistipes* were higher in BH group than those in NC, BL, and BM groups.

Protect effect of the recombinant spores

After the challenge infection with living cercaria (Fig. 5A), *C. sinensis* metacercaria (Fig. 5B) was found in all treated fishes. The average amount of metacercaria per gram fish flesh in the naïve group, B.s-CotC-10^8 group, and B.s-CotC-CsPmy-10^{11} group was 13.2, 12.4 and 7.2, respectively (Fig. 5C).

Discussion

In the current study, we orally administrated grass carp with 1×10^5, 1×10^8 or 1×10^{11} CFU/g *B. subtilis* spores surface displaying CsPmy. The results showed that specific anti-CsPmy IgM levels in sera, skin mucus, intestinal mucus, and bile samples from fishes in B.s-CotC-CsPmy spores orally treated groups dominantly increased. In 1×10^{11} CFU/g B.s-CotC-CsPmy spores administrated fishes, mRNA levels of

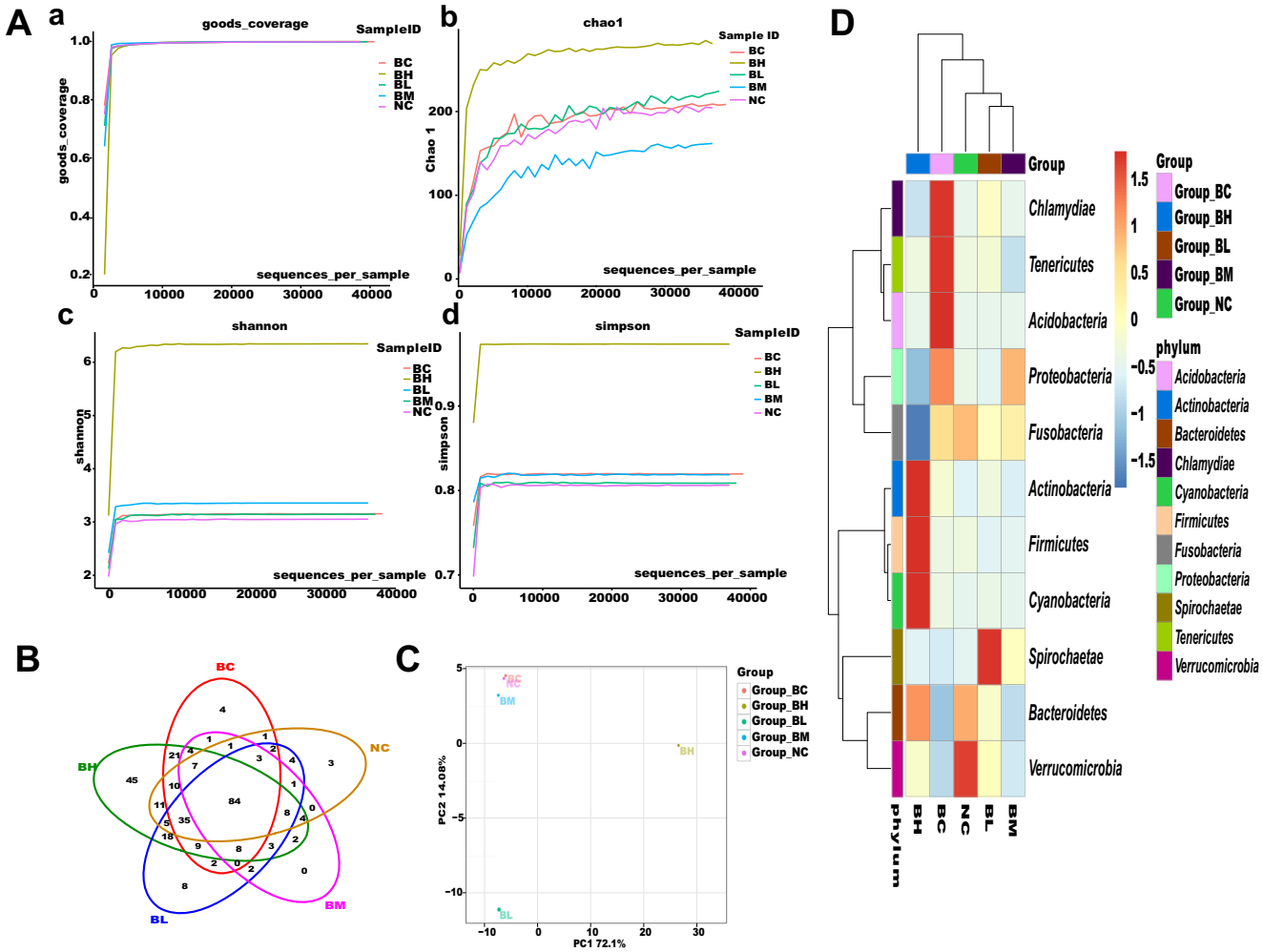

Fig. 4 Intestinal microbiota analysis. Intestines from three fishes in the same group were homogenized for intestinal microbiota analysis. **A** Alpha diversity analysis. **a** Good's nonparametric coverage estimator. **b** Chao1 index. **c** Shannon index. **d** Simpson index. **B** Venn graph showing the unique and shared OTUs among groups. **C** Scatterplot of PCA-score depicting variance of fingerprints derived from the different bacterial community. The principal components PC1 and PC2 explained 72.1% and 14.08% of the variance, respectively. **D** Heatmap of bacterial distribution in groups. The relative abundance of the bacterial genus is depicted by color intensity with legend indicated at the right side of the figure. Clusters based on the distance of five groups along X-axis and bacterial genus along Y-axis were showed in upper and left of the figure, respectively. Naïve, basal diet group; B.s-CotC-10^8: basal diet plus 1×10^8 CFU/g B.s-CotC spores. B.s-CotC-CsPmy-10^5, 10^8, 10^{11}, basal diet plus 10^5, 10^8, 10^{11} CFU/g B.s-CotC-CsPmy spores, respectively

Table 1 Relative abundance of bacteria at phyla level in each group

Groups	BC(%)	BH(%)	BL(%)	BM(%)	NC(%)
Spirochaetae	0.32	1.40	26.37	7.11	1.76
Firmicutes	2.78	25.38	1.20	1.57	3.18
Fusobacteria	17.28	8.20	14.86	16.00	18.23
Proteobacteria	58.55	7.40	21.54	51.15	23.74
Other	0.75	1.91	0.30	0.11	0.19

Numbers represent the relative abundance of the predominant bacterial phyla. NC, fishes were fed with basal diet; BC, fishes were fed with pellets covered with B.s-CotC spores (1×10^8 CFU g^{-1} pellets); BL, BM, BH, fishes were fed with pellets covered with dosages of 1×10^5, 1×10^8, 1×10^{11} CFU g^{-1} pellets of B.s-CotC-CsPmy spores, respectively

IgM, IgZ, TNF-α, and IL-8 significantly elevated in the head kidney and spleen, and transciptional levels of tight-junction proteins in foregut, midgut, and hindgut of grass carp obviously increased too. Gut microbiome indicated that potential pathogenetic bacteria with lower abundance and higher abundances of candidate probiotics and bacteria associated with digestion in 1×10^{11} CFU/g B.s-CotC-CsPmy spores administrated fishes were detected. The amount of metacercaria in per gram fish flesh were statistically decreased in 1×10^{11} CFU/g B.s-CotC-CsPmy spores orally immunized group.

In the past years, the prevention and management of the *C. sinensis* mainly relied on an integrated control strategy including chemotherapy of infected patients, management or

Table 2 Abundance of potential pathogenetic bacteria or candidate probiotics present in samples

Groups	NC	BC	BL	BM	BH
Flavobacterium[*]	6	0	1	0	0
Pseudomonas[*]	128	131	106	103	51
Streptococcus[#]	6	13	9	8	44
Lactobacillus[#]	6	5	2	0	14
Micrococcus[#]	0	0	0	0	0

The numbers represent the total abundance of bacteria genus presented in the community. NC, fishes were fed with basal diet; BC, fishes were fed with pellets covered with B.s-CotC spores (1×10^8 CFU g^{-1} pellets); BL, BM, BH, fishes were fed with pellets covered with dosages of 1×10^5, 1×10^8, 1×10^{11} CFU g^{-1} pellets B.s-CotC-CsPmy spores, respectively

[*] represent the Potential pathogenetic bacteria

[#] the candidate probiotics

sterilization of feces, implementation of education campaigns, etc. (Huang et al. 2017; Lun et al. 2005; Qian et al. 2016). This strategy helped to reduce the infection rate of *C. sinensis* in human beings to a certain degree (Qian et al. 2016), but surveys showed that, in some epidemic areas, the prevalence of *C.sinensis* has been increasing over the years (Lun et al. 2005). Hence, vaccine or more effective drugs are urgently expected to control the spread of *C. sinensis*.

A lot of research works had explored vaccine against *C.sinensis* in recent years, but almost of them were focused on vaccine for the final host (Tang et al. 2016b). To our knowledge, eating raw or undercooked fish flesh harboring living metacercaria is the only route for human being or animals to get infected with *C. sinensis*. In view of this status, we speculated that cutting off the life cycle of *C. sinensis* by interfering the metacercariae formation in freshwater fish might be a potential strategy.

Table 3 Digestion-related bacteria presented in samples

Groups	NC	BC	BL	BM	BH
Rikenellaceae [#]	567	310	414	202	3349
Ruminococcaceae[#]	47	95	50	24	2346
Lachnospiraceae[#]	222	347	294	375	6141
Odoribacter[*]	58	117	64	11	2546
Desulfovibrio[*]	1	2	2	0	19
Alistipes[*]	40	71	50	12	2071

The numbers represent the total abundance of bacteria genus presented in the community. NC, fishes were fed with basal diet; BC, fishes were fed with pellets covered with B.s-CotC spores (1×10^8 CFU g^{-1} pellets); BL, BM, BH, fishes were fed with pellets covered with dosages of 1×10^5, 1×10^8, 1×10^{11} CFU g^{-1} pellets B.s-CotC-CsPmy spores, respectively

[#] at family level

*at genus level

In our previous work, oral vaccines based on *B. subtilis* spore expressing enolase or cysteine protease of *C. sinensis* has already been tested on grass carp and proved to be able to induce both system and local mucosal immune response of fish (Jiang et al. 2017; Tang et al. 2017). But immune protect effect elicited from vaccine candidates besides the two molecules still needs further evaluation and confirmation (Tang et al. 2017). CsPmy, as a multifunctional molecular and important component of cyst wall of *C. sinensis* metacercaria, has been well characterized as a vaccine candidate (Wang et al. 2012). In the current study, CsPmy was displayed on the surface of *B. subtilis* spores by using a coat protein (CotC) as an anchor. SDS-PAGE, western blotting, flow cytometry, and LC-MS/MS verified the successful expression of CsPmy (Fig. 1).

Recombinant *B. subtilis* spores are firstly took in by the M cells in intestinal mucosa and transported into Peyer's patches. Antigens presented on the surface of spores interact with mucosa-associated lymphoid tissues (MALTs) and other systemic lymphoid tissues, resulting in a series of immune responses including secretion of immune globulins (Rosales-Mendoza and Angulo 2015; Tang et al. 2016a; Tavares Batista et al. 2014). In bony fishes, as there are a lot of lymphoid cells, macrophages, eosinophilic, neutrophilic granulocytes, and other secretory cells in skin, it is also a main tissue-producing mucosal immune response besides intestinal mucosa (Salinas 2015). Antigen-specific immune globulins (e.g., IgM and IgZ) were secreted from intestinal mucus and skin mucus when they were stimulated by heterogeneous antigens so that they serve as the first line of immunological barriers against pathogenic invasion (Zhu et al. 2013). Well-studied IgM is the main kind of immune globulins in grass carp. IgZ, analogous to mammalian IgA, also plays a specialized role in mucosal immune responses of fish (Xu et al. 2013).

In the present study, the specific IgM antibody levels in serum, bile, intestinal mucus, and skin mucus of grass carp orally administrated with different dosages of spores (B.s-CotC-CsPmy) were significantly increased with dose-dependent from the 2nd week after the beginning of the immunization till to 6 weeks (Fig. 2). Our results were coincident with the former reports about immune response in grass carp induced by oral immunization with spore expressing enolase or cysteine protease of *C. sinensis* (Jiang et al. 2017; Tang et al. 2017). Moreover, mRNA levels of IgM and IgZ were up-regulated in the head kidney and spleen of fishes from B.s-CotC-CsPmy spores orally treated groups (Fig. 3). The results verified that CsPmy on the spore surface could be recognized by immune system in intestinal mucosa and obviously elicit strong systemic and local mucosal immune response in grass carp by oral administration, especially treated with high dosage (B.s-CotC-CsPmy-10^{11}).

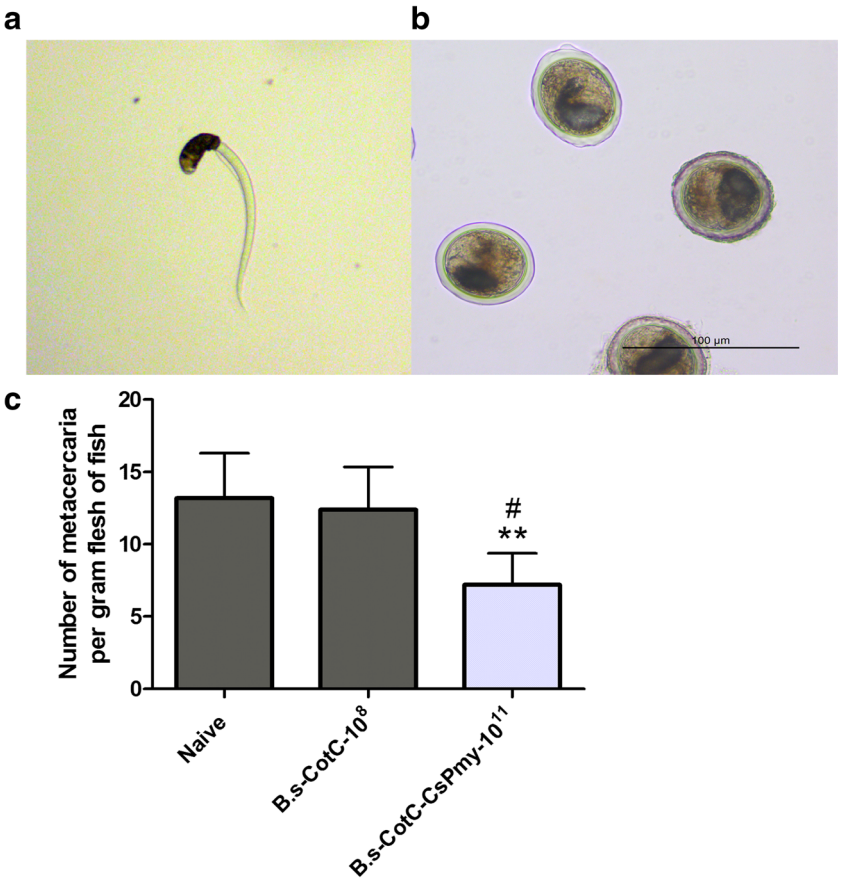

Fig. 5 Protect effect in orally administrated fishes. Fishes were sacrificed at 4 weeks after the challenge infection. Fish flesh in each group was weighted and digested with artificial gastric juice (PH = 2.0) for metacercaria detection. (**A**) *C. sinensis* cercaria used for challenge infection. (**B**) Metacercariae found in grass carp after challenge infection. (**C**) Amount of metacercaria per gram fish flesh. Naïve, fishes fed with basal diet. B.s-CotC-10^8, fishes fed with basal diet plus 1×10^8 CFU/g B.s-CotC spores; B.s-CotC-CsPmy-10^{11}, fishes fed with basal diet plus 10^{11} CFU/g B.s-CotC-CsPmy spores. $^{**}P < 0.01$(compared with Naïve group), $^{\#\#}P < 0.05$ (compared with B.s-CotC-10^8 group). 100 magnification for A and 200 magnification for B

The specific IgM levels in the intestinal mucus and bile of grass carp increased more early than that in sera or skin mucus in B.s-CotC-CsPmy groups (Fig. 2). It might due to that orally delivered antigen (CsPmy) interacted with the local immune system on the intestinal mucosa first and later triggered the systemic immune reaction.

It has been documented that immune status of grass carp was closely related to expression of cytokines (Secombes et al. 2001). IL-8 was considered to be one of the most important proinflammatory cytokines in grass carp (Wang et al. 2013). IL-8 expresses in immune-related tissues in grass carp such as the head kidney, spleen, gill, and so on. IL-8 level would increase when grass carp was stimulated by heterologous antigens or pathogens resulting in attraction of other immune-related cells for fighting against the pathogens (Wang et al. 2013). TNF-α was also proved to be a crucial cytokine involved in immune regulation and mediates the inflammatory responses in grass carp (Zhang et al. 2012; Zhu et al. 2013). In the current study, mRNA levels of TNF-α and IL-8 in the head kidney and spleen in B.s-CotC-CsPmy-10^8 and 10^{11} groups were significantly up-regulated at week 6 (Fig. S1). It demonstrated that oral administration of the recombinant spores activated the innate immunity and might invoke specific adaptive immune responses (Liu et al. 2015). Additionally, the TNF-αlevel had no significant difference in B.s-CotC-CsPmy 1×10^5 and 1×10^8 group in the head kidney. The possible reason might be that the spore dosages (1×10^5 and 1×10^8 CFU/g) were not enough to induce TNF-αsecretion in the head kidney because a certain amount of the spores might be excreted from the intestine with the intestinal movement (Leser et al. 2008).

Studies showed that the structural integrity of intestinal mucosa was crucial to vaccinated fishes (Feng et al. 2015). Tight junction proteins play vital roles in tight junction among epithelial cells of intestine which maintains intestinal integrity resulting in the foundation of mechanical barrier (Chen et al. 2015). Evidences indicated that expressions of tight junction proteins such as ZO-1, occludin, claudin b, or claudin c would be down-regulated when intestinal mucosa was damaged by inflammation (Chen et al. 2015; Feng et al. 2015; Gong et al. 2016). Our results showed that oral administration with 10^8 or 10^{11} CFU/g^{-} *B. subtilis* spores up-regulated mRNA levels of occludin, claudin b, and claudin c in foregut, midgut, and hindgut. mRNA levels of ZO-1 increased in the foregut (Fig. S2). It was previously mentioned that *B. subtilis* could promote epithelial tight junctions of mice with inflammatory bowel disease (Gong et al. 2016), indicating that *B. subtilis* spores were beneficial to maintenance of structural integrity of intestinal mucosa in grass carp.

The gastrointestinal microflora is a complex ecosystem harboring a variety of bacterial communities and plays an imperative role in immune regulation and nutrition consumption of the host (Han et al. 2010; Hao et al. 2017a; Hao et al. 2017b). Variation in the diversity and abundance of bacteria in intestine seriously impairs the immune development and function (Nayak 2010). It has been verified that probiotic additive (e.g., *B. subtilis*) enhance certain innate immune response, thus improve disease resistance of the host (Hao et al. 2017a; Li et al. 2016; Liu et al. 2015). In addition, probiotic supplement in the diets improves growth performance by positively improving the composition of intestinal microbial community (Hao et al. 2017a).

We analyzed intestinal microbiota by using 16S rRNA sequencing. Our results indicated that *Proteobacteria, Firmicutes,* and *Fusobacteria* were the dominant phyla in each group (Table 1), suggesting that dietary supplement of *B. subtilis* spores did not affect the primary phyla constitution of grass carp. Compared with naïve group, both the diversity and richness indices of bacteria in intestines of spores administrated grass carp were elevated, especially in B.s-CotC-CsPmy-10^{11} group. The results of PCA, Venn, and heatmap also verified that the intestinal microbiota in B.s-CotC-CsPmy-10^{11} group was dramatically different from the other groups (Fig. 4).

Previous studies reported that *B. subtilis* Ch9 and *B. subtilis* CGMCC 1.1086 strains could increase probiotics but decrease pathogenetic bacteria in intestinal of grass carp or broilers (Hao et al. 2017a; Li et al. 2016). *Streptococcus, Lactobacillus,* and *Micrococcus* have been widely used as probiotics in aquaculture and brought positive effects on the host including improvement of the immune status and inhibiting pathogen infection in the intestines (Balcazar et al. 2006; Hai 2015). In our present study, the abundance of *Streptococcus* and *Lactobacillus* in high-dosage spores-treated group was dramatically higher than those in other groups (Table 2), while *Lactobacillus* was not detected in intestine of *B. subtilis* treated fish (Hao et al. 2017a). Considering that culture condition and feed may affect intestinal microbiota of fish (Han et al. 2010; Wu et al. 2012), it might due to the different culture condition and diet in different experiments.

Some strains from *Flavobacterium* and *Pseudomonas* genera were considered to be potential pathogens to grass carp, as they might induce columnaris disease, white head-mouth disease, and red skin disease (Hao et al. 2017a). Lower abundance of *Flavobacterium* was found in naïve group but not in *B. subtilis* treated groups. Dramatically lower abundance of *Pseudomonas* in B.s-CotC-CsPmy-10^{11} group was detected compared with Naïve group (Table 2). The results demonstrated that oral administration with probiotics might reduce potential pathogenic bacteria in grass carp intestine (Hao et al. 2017a; Liu et al. 2015). A possible explanation might be that probiotics generate antibiotics, bacteriocins, hydrogen peroxide, and lysozyme, etc., thus inhibit the adherence or proliferation of pathogens in intestinal mucosa (Hai 2015; Sugita et al. 1998).

In addition, *Rikenellaceae, Ruminococcaceae,* and *Lachnospiraceae* were dramatically increased in B.s-CotC-CsPmy-10^{11} group. At genus level, higher abundance of *Alistipes, Odoribacter,* and *Desulfovibrio* in the high-dosage group were presented (Table 3). *Rikenellaceae* were considered as intestine beneficial bacteria, as *Alistipes* genus belong to the family can produce fibrinolysin, digest gelatin, and ferment carbohydrate resulting in enhancement of digestion in fishes(Li et al. 2016). *Odoribacter* can produce short-chain fatty acids (SCFAs) such as acetic acid, succinic acid, or butyric acid, which are beneficial to both microbial and epithelial cell growth of host (Meehan and Beiko 2014). *Desulfovibrio* has also been proved to be beneficial to the microbial community and able to improve energy recovery from food (Li et al. 2016). *Lachnospiraceae* are a butyrate-producing superfamily, which can produce an enzyme to break down a wide variety of complex carbohydrates found in plants (Hao et al. 2017b). Hence, higher abundances of these bacteria found in the intestines would probably be helpful to digestion function of grass carp and promote their growth performance.

Compared with naïve group, abundances of bacteria mentioned above were not obviously changed in BC, BL, and BM group (B.s-CotC-10^{8}, B.s-CotC- CsPmy-10^{5}/10^{8} groups) (Tables 1, 2, 3). It might because that lower dosage of *B. subtilis* spores accompanied by less colonized *B. subtilis* spores in intestine of grass carp (Hao et al. 2017a).

After oral immunization, the fishes were infected with *C. sinensis* cercaria for a week. Compared with naïve group, metacercaria intensities in B.s-CotC-CsPmy-10^{11} spore treated grass carp were significantly decreased (Fig. 5C). We conjectured that immune responses induced by the recombinant spores (B.s-CotC-CsPmy) played roles in protecting against adherence or invasion of cercaria to grass carp. It needed further study to illuminate the involved mechanisms. When grass carp was orally administrated with B.s-CotC-CSCP spore (10^{7} dosages), no metacercariae were observed (Tang et al. 2017). We speculated that might be due to two reasons: firstly, the function and immunogenicity of CsPmy were different from those of CsCP (Tang et al. 2016a; Wang et al. 2012) resulting in different protect effect. Secondly, the methods used for metacercariae detection were different. In the former study, metacercariae were checked by direct compression method by randomly picking fish flesh from 5 different positions in one fish (Tang et al. 2017), which might lead to missed detection when the infection intensity was low. While in our present study, we checked by digestion method with high detection rate. That is, the fishes were totally digested with artificial gastric juice, so that every metacercariae could be detected.

In conclusion, oral administration with B.s-CotC-CsPmy spores could induced both systemic and local mucosal immune responses without bad effects on intestinal structural integrity and elicited promising protective effect in grass carp. Additionally, oral treatment of the spores in grass carp could reduce the abundance of potentially pathogenic bacteria (e.g., *Flavobacterium*) and enhance the abundance of probiotics (e.g., *Streptococcus, Lactobacillus*) or bacteria associated with digestion. Our work suggested that *B. subtilis* spore presenting CsPmy on the surface is a promising effect, safe, and needle-free oral vaccine candidate for prevention of *C. sinensis* infection in grass carp. Our study established the cornerstone for the prevention of *C. sinensis* infection in both human and mammalian reservoir hosts by using fish vaccine to cut off its life cycle. This work also shed light on the vaccine development for other zoonosis.

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Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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