

Effect of dietary probiotic supplementation on intestinal microbiota and physiological conditions of Nile tilapia (*Oreochromis niloticus*) under waterborne cadmium exposure

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Abstract The heavy metal cadmium (Cd) is a hazardous pollutant that exerts various toxic effects on aquatic animals. The biomagnifying effects of this non-essential element in the food chain also pose threats to human health. In this study, the protective effect of a dietary probiotic supplementation, *Lactobacillus plantarum* CCFM8610, on the intestinal microbiota and physiological conditions of Nile tilapia (*Oreochromis niloticus*) exposed to waterborne Cd was evaluated. Two hundred fish were divided into four groups, i.e., control, probiotic-only, Cd-only and Cd-plus-probiotic. The fish were exposed to

waterborne Cd at a level of 1 mg L^{-1} for 4 weeks and the probiotic was administered twice daily at 10^8 CFU g^{-1} in the fish diet. Waterborne Cd exposure caused a profound decline in the gut microbial diversity and marked alterations in the composition of the microbiota. Dietary supplementation with *L. plantarum* CCFM8610 reversed the changes in the intestinal microbiota composition in the Cd-exposed fish and reduced the abundance of *Flavobacterium* and *Pseudomonas*. Compared with the Cd-only group, the probiotic treatment significantly promoted growth performance and prevented the death of the Cd-exposed fish. *L. plantarum* CCFM8610 supplementation also decreased Cd accumulation and alleviated oxidative stress in the tissues, and reversed the alterations in hemato-biochemical parameters in the blood of fish. The results suggest that *L. plantarum* CCFM8610 can be considered a safe dietary supplement for the prevention of Cd-exposure-induced problems in aquaculture and food safety.

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Introduction

Cadmium (Cd) is a non-essential element that can be toxic and carcinogenic to humans and animals. This heavy metal currently ranks seventh on the priority list

of hazardous substances provided by the Agency for Toxic Substances and Disease Registry (ATSDR 2015) of the United States. With industrial development and population growth, increasing contamination of aquatic systems with Cd has been reported worldwide (Chahid et al. 2014; Taweel et al. 2013; Zhou et al. 2008). Through exposure to Cd present in water, sediments and the food chain, this toxic metal can be readily assimilated and bioaccumulated in a variety of aquatic organisms, such as Nile tilapia (*Oreochromis niloticus*), shrimp (*Parapenaeus longirostris*), mussel (*Mytilus edulis*) and octopus (*Octopus vulgaris*) (Morgano et al. 2014; Oimedo et al. 2013; Taweel et al. 2013). Cd exposure causes oxidative stress and immunotoxicity in fish, which induces structural and functional disorders in vital organs including the gills, liver and kidneys, leading to growth inhibition and abnormal mortality (Almeida et al. 2002; Guardiola et al. 2013). Therefore, Cd pollution in the aquatic environment not only causes great economic losses in aquaculture, but also poses potential human health risks as a result of aquatic product consumption (Copat et al. 2013; Kumar and Singh 2010; Oimedo et al. 2013). Thus, the development of safe, economic and efficient strategies to control Cd levels in fish is an area of ongoing research (Schwarzenbach et al. 2006; Zhou et al. 2008).

The intestinal microbiota of fish plays an important role in immunity, metabolism, maturation and pathogen resistance (Gómez and Balcázar 2008). Imbalances of the microbiota may cause disturbances of the intestinal immune system and contribute to the development of diseases in fish (Pérez et al. 2010). Cd exposure has been reported to significantly affect the intestinal microbiota of mammals (Bisanz et al. 2014; Liu et al. 2014), but the effects of this toxic metal on the gut microbiota of fish are not well understood.

The application of probiotics in aquatic feeds has been studied extensively and is accepted as an alternative to reduce the misuse of antibiotics (Gatesoupe 1999). A considerable number of studies have also confirmed that probiotic supplementation can effectively modulate the intestinal microbiota, promote growth performance and regulate immune homeostasis in fish (Heo et al. 2013; Standen et al. 2013, 2015). In our previous studies, a specific probiotic, *Lactobacillus plantarum* CCFM8610, was screened out for its useful Cd binding and anti-

oxidative stress ability both in vitro and in vivo (Zhai et al. 2014, 2015). Preliminary experiments for the present study also showed that dietary supplementation with this strain significantly decreased the mortality (4/20 to 0/20) and muscle Cd level (decreased by more than 50%) of Cd-exposed Nile tilapia. These results indicated the potency of this lactic acid bacterium for the treatment or prevention of Cd exposure in aquaculture. 16S rRNA gene sequence analysis confirmed the identification of the strain as *L. plantarum* (99% sequence identity to *L. plantarum* AJ965482).

Therefore, the aims of this study were to investigate the effects of dietary *L. plantarum* CCFM8610 supplementation on the intestinal microbiota and physiological conditions of Nile tilapia exposed to waterborne Cd, and to gain insights into the possible protective mechanisms of this probiotic.

Materials and methods

Bacterial strains and culture

L. plantarum CCFM8610 was obtained from the in-house Culture Collections of Food Microbiology (CCFM), Jiangnan University (Wuxi, China). The strain was cultured in de Man-Rogosa-Sharpe (MRS) broth (Hopebio Company, China) at 37 °C for 18 h.

Preparation of the fish diet

The fish diet (crude protein 32%, crude lipid 8%, crude fiber 10% and ash 12%) was formulated as previously reported, with minor modifications (Ma et al. 2015). For the preparation of the experimental floating feed, the *L. plantarum* CCFM8610 culture was suspended in phosphate saline buffer (pH 7.2) and then mixed with a weighed amount of fish diet powder at an initial bacterial concentration of 10^{10} CFU g⁻¹, which corresponded to a bacterial level of 10^8 CFU g⁻¹ after pelleting. The dose of the *L. plantarum* strain was selected based on previous reports (Heo et al. 2013; Ridha and Azad 2016). The bacterial concentration in the fish diet was also confirmed by colony counting. The probiotic-containing feed was prepared weekly and stored at 4 °C. Based on our preliminary colony-counting experiment with an incubation on MRS agar

at 37 °C for 48 h, the viability of *L. plantarum* CCFM8610 in the feed was confirmed to remain at the level of 10^8 CFU g^{-1} during the 1-week storage.

Fish and experimental design

Nile tilapia were obtained from the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences (Wuxi, China) with a health certificate. The fish were acclimated in cylindrical plastic tanks for 3 weeks at 28 ± 0.3 °C, pH 7.6 ± 0.2 , dissolved oxygen $7.2\text{--}7.8$ mg L^{-1} , with a 12-h light–dark photoperiod and continuous aeration. No death or abnormality was observed during the acclimation.

After acclimation, 200 fish with an average body weight of 34.0 ± 1.16 g were randomly divided into four groups, i.e., control, CCFM8610-only, Cd-only and Cd-plus-CCFM8610, with triplicate tanks per group (16 or 17 fish per tank). Fish in the control group received basal fish diet and were kept in Cd-free water. Fish in the CCFM8610-only group received diet containing *L. plantarum* CCFM8610 (10^8 CFU g^{-1}) and were kept in Cd-free water. Fish in the Cd-only group received basal fish diet and were exposed to waterborne Cd at 1 mg L^{-1} . Fish in the Cd-plus-CCFM8610 group were treated with both probiotic-containing diet and waterborne Cd exposure. A dose of waterborne Cd at a level of 1 mg L^{-1} , in the form of $CdCl_2$, and a feeding period of 4-week were selected to model environmental Cd exposure with sublethal toxic effects on fish (Almeida et al. 2001; Franklin et al. 2005; Guardiola et al. 2013). Our preliminary experiment also showed that this feeding regime exhibited a moderate toxic effect on fish with a mortality at 20% and a muscle Cd level at 0.12 ± 0.031 μg g^{-1} of wet tissue. The fish were fed with the experimental floating feed at 3% of their body weight twice daily (9:00 a.m. and 5:00 p.m.). Care was taken to avoid feed losses during the experiment. The water was refreshed every 2 days to maintain a constant Cd level and the faeces of the fish were collected and siphoned off daily. The water quality was monitored throughout the trial and the experiments were conducted under the same conditions as in the acclimation period. Water samples were collected every 24 h for Cd level determination using a flame or graphite furnace atomic absorption spectrophotometer (Spectr AAS or AA; Varian).

During the 4-week trial, the body weight (BW) and feed intake of the fish were recorded and the growth performance was evaluated by calculating the growth rate (GR), feed conversion ratio (FCR) and survival rate as follows (Ma et al. 2015).

$$GR(\%) = [(BW_f - BW_i)/BW_i] \times 100\%$$

$$FCR = \text{Total feed intake}/(BW_f - BW_i)$$

$$\text{Survival rate}(\%) = (N_s/N_i) \times 100\%$$

where BW_f and BW_i are the final BW of the fish after the 4-week treatment period and the initial BW of the fish, respectively. N_s and N_i are the number of surviving fish after the 4-week treatment period and the initial number of fish, respectively.

At the end of the 4th week, faecal samples were collected from each tank and stored at -80 °C. The fish were then sacrificed under ethyl 3-aminobenzoate methanesulfonate anesthesia. Blood was collected from the caudal vasculature of five fish per tank (15 replicates per group) and divided into two aliquots: one was centrifuged (3000g, 10 min) to obtain serum samples and the other was stored in anti-coagulative tubes (EDTA-2K). Tissue samples including spleen, brain, kidney, liver, gill, gut and muscle were collected from three fish per tank (nine replicates per group) and stored in metal-free Eppendorf tubes at -80 °C. The intestinal contents were squeezed into a sterilised tube and stored at -20 °C for further microbial analysis after the removal of the gut.

All of the protocols for this study were approved by the Ethics Committee of Jiangnan University, China (JN no. 2015-09-F). The procedures of this study involving fish were carried out in accordance with the European Community guidelines (directive 2010/63/EU) for the care and use of experimental animals.

Analysis of gut microbial diversity and quantification of faecal *L. plantarum*

As very few gut contents could be collected from the fish in the Cd-only group, the samples were pooled by tank (thus $n = 3$ per treatment) and microbial DNA was extracted from 200 mg gut content samples using the E.Z.N.A.[®] DNA Kit (Omega Bio-tek, Norcross, GA, US) following the manufacturer's instructions. The microbial DNA from faecal samples was

extracted with the same kit for the quantification of *L. plantarum*.

The V4–V5 region of the bacterial 16S ribosomal RNA gene was amplified by PCR using the primers 515F (5'-barcode-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTC AATTCMTTTRAGTTT-3'), where the barcode is an eight-base sequence and unique to each sample. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, US) and quantified using QuantiFluorTM-ST (Promega, US). The purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP089871).

The raw fastq files were de-multiplexed and quality-filtered using QIIME (version 1.17). Operational units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (<http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analysed using the RDP Classifier (<http://rdp.cme.msu.edu/>) against the SILVA (SSU115) 16S rRNA database using a confidence threshold of 70% (Amato et al. 2013).

Primers (LP-F, 5'-GGAGCCGCTATTAGTATTTTCAT-3' and LP-R 5'-AATACAAGCAAGTCTTGGACCAG-3'), specific for *L. plantarum*, were used to quantify the level of this species in the faeces as previously reported, with minor modifications (Costa et al. 2014; Klocke et al. 2006). The quantitative PCR (qPCR) analysis was performed in a reaction mixture (20 µL) containing 2 × SYBR Green Supermix (Bio-Rad, US), 1 µM of each primer and 1 µL of the microbial DNA extracted from the faecal samples. A dissociation curve analysis was then performed and the cycle threshold (Ct value) of each sample was compared with the corresponding standard curve to determine the number of gene copies of *L. plantarum* (Million et al. 2012; Romi et al. 2015).

Determination of hemato-biochemical parameters in blood

Total white blood cell (WBC) and red blood cell (RBC) counts were determined using a Neubauer hemocytometer (BC-5300V; Mindray, China)

(Gabriel et al. 2015). The levels of hematocrit percentage (HCT %) and hemoglobin (HGB) were measured as previously described (Gabriel et al. 2015). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated as follows (Gabriel et al. 2015).

$$\text{MCV} = (\text{HCT} \times 10) / \text{RBC}$$

$$\text{MCH} = (\text{HGB} \times 10) / \text{RBC}$$

$$\text{MCHC} = (\text{HGB} \times 100) / \text{HCT}$$

Biochemical parameters in the serum of the fish, including the total cholesterol (TC), total protein content (TP), triglyceride (TG), high-density lipoprotein (HDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured using an automatic biochemical analyser (BS-400; Mindray, China) (Ma et al. 2015).

Determination of Cd and other chemical elements in tissues

The tissue samples were transferred to metal-free digestion vessels (OMNI; CEM, UK) and digested in concentrated HNO₃ using a microwave digestion system (MARS; CEM, UK). The Cd concentrations in all of the tissue samples and the Cd, calcium (Ca), iron (Fe), magnesium (Mg) and zinc (Zn) levels in the muscles were determined by inductively coupled plasma mass spectrometry (NexIon-300X; PerkinElmer, China).

Determination of oxidative stress levels in tissues

The levels of glutathione (GSH), of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the brain, liver and kidneys of the fish were measured with commercial assay kits (Jiancheng Bioengineering Institute, China). The assays were performed according to the recommendations of the manufacturer.

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) for each group. The differences among the groups were evaluated by one-way analysis

of variance, followed by the Tukey post hoc test. A P value of <0.05 was considered to be statistically significant.

Results

Fish growth and survival performance

Waterborne Cd exposure without probiotic supplementation (Cd-only) caused a marked inhibition of growth and feed utilisation, and a mortality of 6 of the 50 tilapia (Table 1). Compared with the Cd-only group, dietary supplementation with *L. plantarum* CCFM8610 significantly increased the GR and decreased the FCR ($P < 0.05$), and completely prevented the death of Cd-exposed fish. The growth performance and feed utilisation of the tilapia fed *L. plantarum* CCFM8610 without Cd exposure (probiotic-only) were also significantly promoted compared with those in the control group ($P < 0.05$). As shown in Table S1, the Cd levels in the aquatic environment of the Cd-only group decreased after 24 and 48 h, which may be due to the uptake by fish and the natural sedimentation. Water Cd levels in the Cd-plus-*L. plantarum* group were lower than that in the Cd-only group.

Intestinal microbial diversity and composition

Cd exposure caused a profound decline in the gut microbial diversity and marked alterations in the composition of the microbiota. At the phylum level (Fig. 1), Cd exposure resulted in a significant increase in *Bacteroidetes* and a considerable decrease in *Fusobacteria*. At the genus level, both Cd exposure

and *L. plantarum* CCFM8610 treatment altered the structure and composition of the fish gut microbiota (Fig. 2). 152, 149, 116 and 103 classified genera were detected in the control, CCFM8610-only, Cd-only and Cd-plus-CCFM8610 groups, respectively. Compared with the Cd-free groups, Cd exposure caused profound decreases in *Cetobacterium*, *Plesiomonas* and *Deefgea* and significantly increased the abundance of *Flavobacterium*, *Pseudomonas*, *Cellvibrio* and *Acinetobacter* (Fig. 3, $P < 0.05$). Dietary supplementation with *L. plantarum* CCFM8610 reduced the abundance of *Flavobacterium* ($P = 0.093$) and *Pseudomonas* ($P = 0.061$) in the Cd-exposed fish. Compared with the control group, *L. plantarum* CCFM8610 treatment by itself resulted in a significant increase in the abundance of *Deefgea* ($P = 0.016$). The qPCR assay demonstrated that the supplementation with the probiotic-containing diet markedly increased the concentration of *L. plantarum* in the faeces of the fish, while Cd exposure caused a reduction in the *L. plantarum* population in the 4th week (Table 2, $P < 0.05$).

Cd, Ca, Fe, Mg and Zn levels in the tissues and blood

The Cd levels detected in the blood, spleen, brain, kidneys, liver, gills, gut and muscles of the tilapia are shown in Tables 3 and 4. Compared with the control group, waterborne Cd exposure significantly increased the levels of this toxic metal in the blood and tissues ($P < 0.05$), and the kidneys and liver were the organs with the greatest Cd accumulation. *L. plantarum* CCFM8610 supplementation significantly decreased the Cd levels in the blood, spleen, kidneys, gills and muscles of the fish ($P < 0.05$). The Cd levels in the gut were higher in the Cd-plus-CCFM8610 group than in

Table 1 Effects of dietary *L. plantarum* CCFM8610 supplementation on growth performance of Nile tilapia during the 4-week experiment

Treatment (group)	GR (%)	FCR	Survival rate (%)
Control	29.69 ± 0.52 ^a	1.98 ± 0.03 ^a	100
8610 only	35.47 ± 1.24 ^b	1.70 ± 0.06 ^b	100
Cd only	2.63 ± 0.25 ^c	18.21 ± 0.05 ^c	88
Cd + 8610	18.59 ± 1.28 ^d	2.80 ± 0.01 ^d	100

GR relative growth rate, FCR feed conversion ratio. Data are expressed as the mean ± SEM for each group. The superscript letters indicate statistically significant differences at a P value of <0.05 within each row comparison

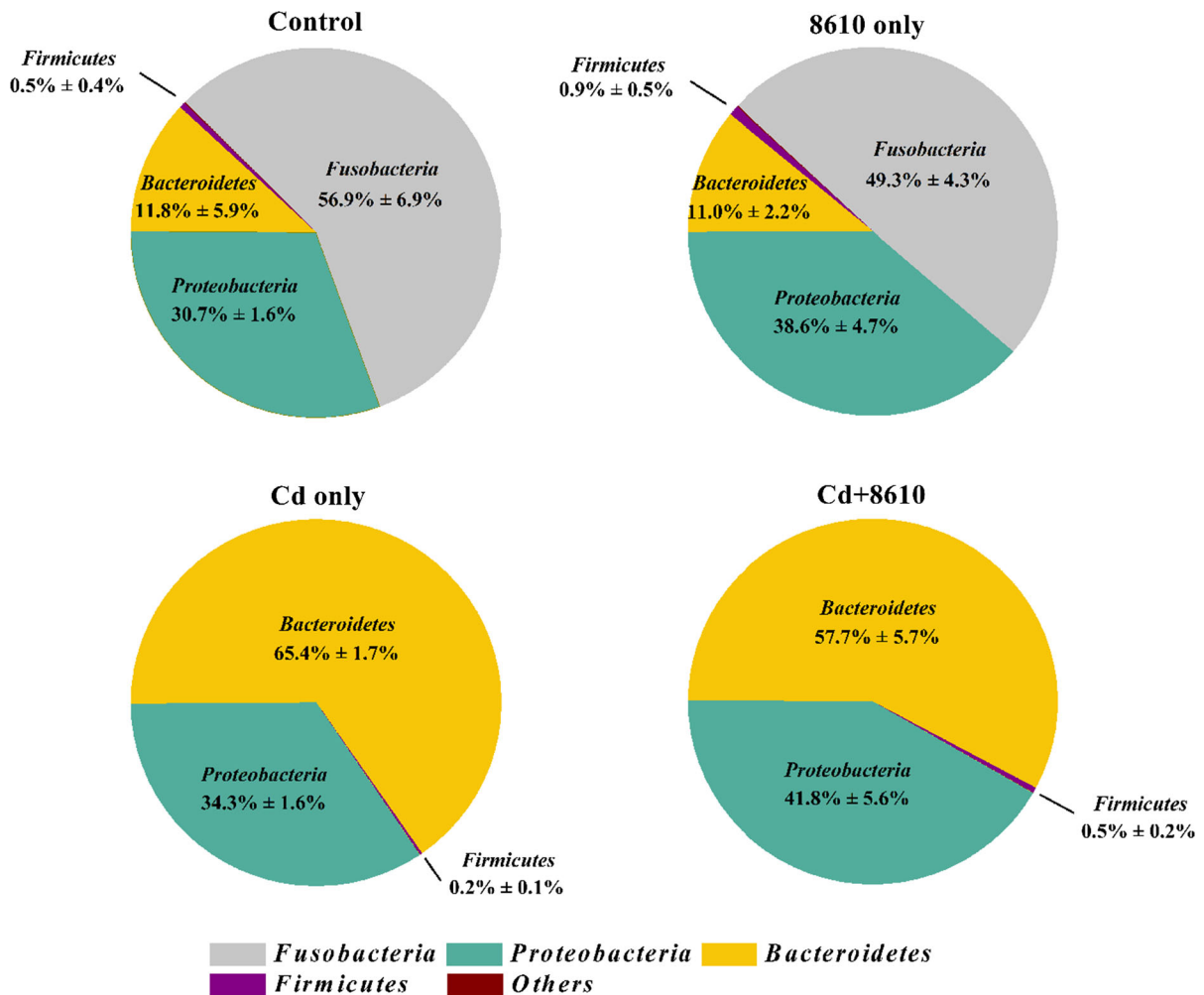


Fig. 1 Effects of waterborne Cd exposure and dietary *L. plantarum* CCFM8610 supplementation on the relative abundance of the main phyla of the gut microbiota of Nile tilapia. Data are expressed as the mean \pm SEM for each group

the Cd-only group. Cd exposure also caused alterations of the Ca and Zn levels in the muscles of the fish (Table 4). These alterations were not reversed in the Cd-plus-CCFM8610 group ($P > 0.05$). *L. plantarum* CCFM8610 treatment by itself did not result in significant differences in the levels of any of the metals from those in the control group, with the exception of an increase in the Zn level in the muscles.

Hemato-biochemical parameters in blood

Cd exposure significantly decreased the levels of the hematological parameters HCT, HGB, MCV and WBC (Figures S1 and S2). Compared with the Cd-

only group, the decreases in the HGB and WBC levels were markedly reversed by *L. plantarum* CCFM8610 treatment ($P < 0.05$), while the other parameters remained unaffected. As shown in Figure S3, Cd exposure also caused significant alterations of the blood biochemical parameters TC, TG, TP, ALT, AST and HDL ($P < 0.05$). Dietary supplementation with *L. plantarum* CCFM8610 markedly recovered the levels of TG, ALT and AST ($P < 0.05$). We also noted that CCFM8610 treatment by itself did not result in significant differences in the levels of the hemato-biochemical parameters from those in the control group, with the exception of enhancements of the HGB and HDL levels.

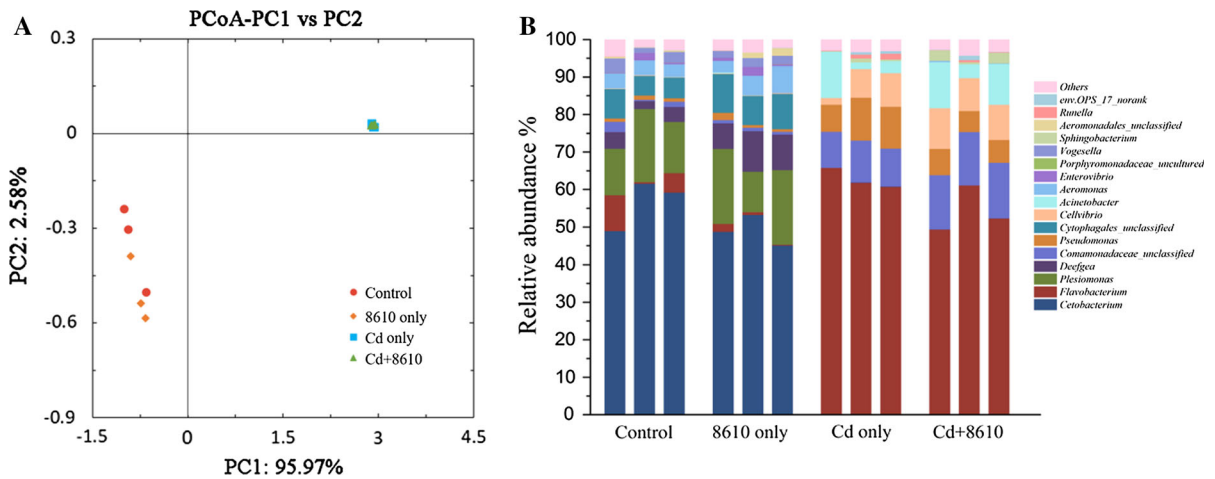


Fig. 2 Effects of waterborne Cd exposure and dietary *L. plantarum* CCFM8610 supplementation on the gut microbial compositions of Nile tilapia. **a** Principal coordinate (PCoA) score plots based on unweighted UniFrac metrics. Indication of

principal coordinate percent variation is next to each axis. Each *point* represents the composition of the gut microbiota of one tank of fish. **b** Effects of Cd and probiotic treatments on the gut microbial compositions of Nile tilapia at the genus level

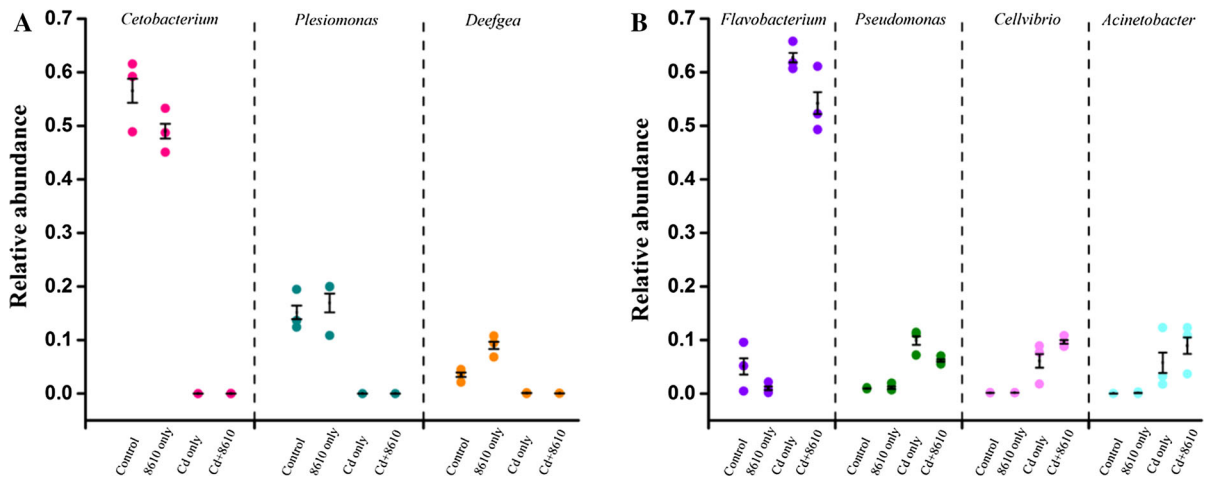


Fig. 3 Effects of waterborne Cd exposure and dietary *L. plantarum* CCFM8610 supplementation on the changes in the bacterial species of interest. **a** Relative abundance of

Cetobacterium, *Plesiomonas* and *Deefgea*. **b** Relative abundance of *Flavobacterium*, *Pseudomonas*, *Cellvibrio* and *Acinetobacter*. Data are expressed as the mean \pm SEM for each group

SOD, GPx, GSH and MDA in the tissues

The activities of SOD and GPx and the level of GSH in the livers of the fish (Figure S4) were significantly reduced in the Cd-only group, accompanied by a marked increase in the levels of MDA ($P < 0.05$). Similar trends were also observed in the brains of the fish (Figure S5) i.e., Cd exposure inhibited the activities of SOD and GPx and increased the level of MDA. Compared with the control group, all of

these parameters remained unaffected in the group treated with *L. plantarum* CCFM8610 only. Compared with the fish in the Cd-only group, *L. plantarum* CCFM8610 supplementation was effective in reversing the alterations in GPx and MDA in the liver and the changes in SOD and MDA in the brain, respectively. Neither Cd exposure nor probiotic treatment caused significant alterations in these oxidative stress-related parameters in the kidneys of the fish (data not shown).

Table 2 Quantification of *L. plantarum* population in the faeces of Nile tilapia during the 4-week experiment by qPCR assay

Treatment (group)	Log ₁₀ gene copies/g faecal sample ± SEM at		
	0 week	2nd week	4th week
Control	5.27 ± 0.06 ^a	5.43 ± 0.03 ^a	5.23 ± 0.03 ^a
8610 only	5.46 ± 0.13 ^a	7.76 ± 0.03 ^b	7.55 ± 0.04 ^b
Cd only	5.33 ± 0.02 ^a	5.32 ± 0.04 ^a	4.71 ± 0.04 ^c
Cd + 8610	5.62 ± 0.21 ^a	6.93 ± 0.02 ^c	6.95 ± 0.05 ^d

Data are expressed as the mean ± SEM for each group. The superscript letters indicate statistically significant differences at a *P* value of <0.05 within each row comparison

Discussion

The heavy metal Cd is a hazardous pollutant that has various toxic effects on aquatic animals. The biomagnifying effects of this non-essential element in the food chain also pose challenges to public human health. Motivated by the previously identified potential of a specific probiotic (*L. plantarum* CCFM8610) against Cd toxicity, this study investigated the use of diet containing this probiotic for protection against waterborne Cd exposure in fish.

Based on previous reports (Kumar and Singh 2010; Wright and Welbourn 1994), the cycle of Cd in the aquatic ecosystem is illustrated in Fig. 4. Besides being primarily taken up by the gills, waterborne Cd can also contaminate the diet and be absorbed via the intestines of fish (Wang et al. 2012). After absorption, Cd accumulates in the tissues and can be excreted by the kidneys, liver, intestine and gills, thus recycling through the aquatic system (Kumar and Singh 2010).

L. plantarum CCFM8610 has been reported to effectively bind Cd in vitro and sequester Cd in the intestines of mice (Zhai et al. 2014, 2015). This strain is able to bind dissociated aqueous Cd ions in vitro and sequester foodborne Cd in vivo, thus preventing this toxic metal from being absorbed via the gills and intestines of fish. Moreover, a portion of the Cd accumulated in the liver can be excreted into the gut via hematic and enterohepatic circulation and be re-absorbed by the intestines efficiently (Nordberg et al. 2011; Roberts et al. 2002). Therefore, intestinal *L. plantarum* CCFM8610 could bind and immobilise such secreted Cd before the intestinal re-absorption, due to its superior Cd binding ability (Zhai et al. 2013). This may explain the higher Cd level in the gut of the Cd-plus-CCFM8610 group than that of the Cd-only group (Table 3). Dietary supplementation with this

strain can therefore increase faecal Cd excretion, because lactic acid bacteria are excreted through the faeces of fish (Ringø and Gatesoupe 1998). As the faecal Cd is sequestered by the strain, *L. plantarum* CCFM8610 can also prevent the recycling of Cd into the aquatic system, thus reducing the risk of re-exposure of fish. This is confirmed by a lower water Cd level in the Cd-plus-*L. plantarum* group than that in the Cd-only group (Table S1). These mechanisms (Fig. 4) may explain the significant effects of dietary *L. plantarum* CCFM8610 supplementation against Cd accumulation in the tissues and blood of fish (Tables 3, 4).

To the best of our knowledge, very few studies on the toxic effects of Cd exposure on fish gut microbiota have been carried out to date. The present study showed that waterborne Cd exposure profoundly affected the diversity and composition of the gut microbiota of Nile tilapia (Figs. 1, 2, 3). Among the bacteria that were significantly decreased after Cd exposure, *Cetobacterium* is a predominant genus in the intestines of freshwater fish (Larsen et al. 2014) and *Cetobacterium somerae* has been reported to be significant in vitamin B₁₂ synthesis in fish (Tsuchiya et al. 2008). Members of the genera *Plesiomonas* and *Deefgea* have also been found to commonly inhabit the intestinal tracts of fish (Herrera et al. 2006; Jung and Jung-Schroers 2011). Among the bacteria that were markedly increased after Cd exposure, *Flavobacterium* strains have been reported to be pathogenic to fish: *Flavobacterium psychrophilum* is the pathogen responsible for bacterial cold water disease and *Flavobacterium columnare* causes the disease columnaris in several freshwater fish species (Leal et al. 2010; Nematollahi et al. 2003). Some strains of the genera *Pseudomonas* and *Acinetobacter* may also exert adverse effects on fish health:

Table 3 Effects of dietary *L. plantarum* CCFM8610 supplementation on Cd levels in the blood, spleen, brain, kidneys, liver, gills and gut of Nile tilapia

Treatment (group)	Mean concentration ($\mu\text{g g}^{-1}$ of blood or wet tissue) \pm SEM						
	Blood	Spleen	Brain	Kidney	Liver	Gill	Gut
Control	0.004 \pm 0.002 ^a	0.01 \pm 0.004 ^a	–	0.62 \pm 0.092 ^a	0.37 \pm 0.017 ^a	0.017 \pm 0.002 ^a	0.19 \pm 0.040 ^a
8610 only	0.003 \pm 0.001 ^a	0.01 \pm 0.003 ^a	–	0.59 \pm 0.095 ^a	0.38 \pm 0.030 ^a	0.017 \pm 0.002 ^a	0.13 \pm 0.027 ^a
Cd only	0.34 \pm 0.059 ^b	6.19 \pm 0.41 ^b	7.44 \pm 0.49 ^b	116.00 \pm 7.31 ^b	235.56 \pm 9.73 ^b	8.01 \pm 0.38 ^b	69.38 \pm 4.77 ^b
Cd + 8610	0.19 \pm 0.022 ^c	4.43 \pm 0.27 ^c	7.01 \pm 0.30 ^b	86.44 \pm 12.22 ^c	211.11 \pm 9.49 ^b	6.41 \pm 0.34 ^c	117.78 \pm 6.02 ^c

–, Cd concentration in the tissue was too low to detect. Data are expressed as the mean \pm SEM for each group. The superscript letters indicate statistically significant differences at a *P* value of <0.05 within each row comparison

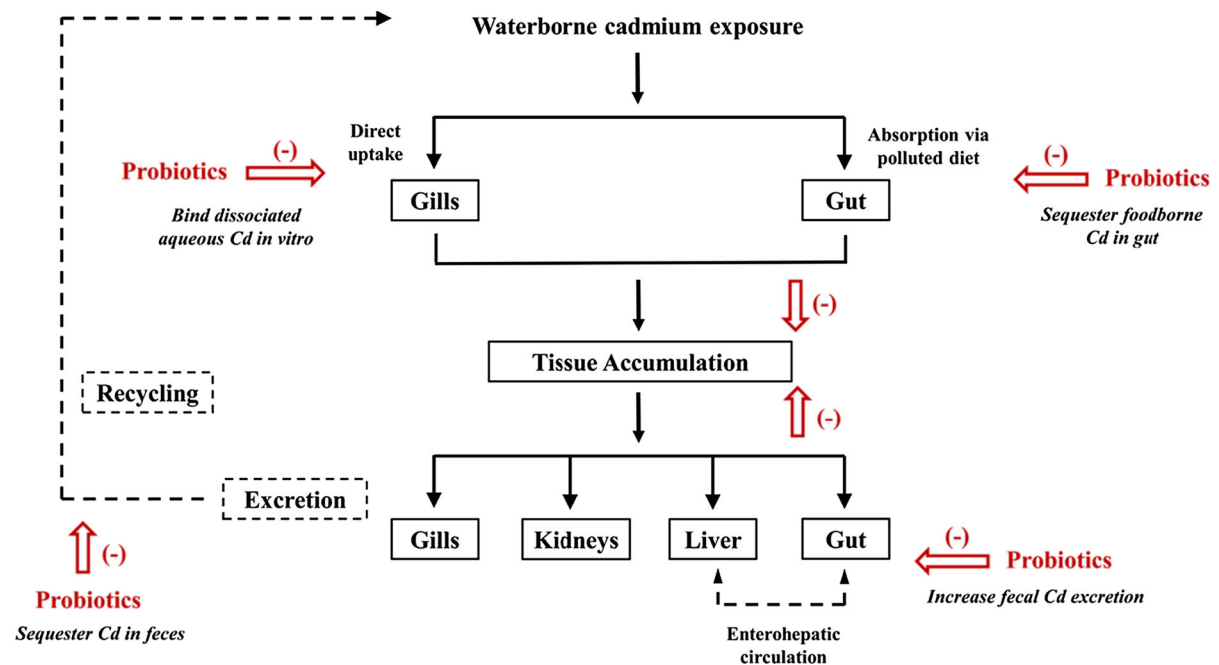
Pseudomonas plecoglossicida and *Pseudomonas anguilliseptica* infections in ayu and salmonid fish have been reported in Japan and Finland, respectively (Park et al. 2000; Wiklund and Bylund 1990), and *Acinetobacter baumannii* has been identified as a pathogen for channel catfish in China (Xia et al. 2008). The gut microbiota is in continuous direct contact with the intestinal mucosa and plays an important role in fish health (Pérez et al. 2010). Cd-induced alterations in the gut microbiota may cause severe dysfunctions in Nile tilapia, which can be another possible cause of the adverse effects observed on the growth performance, antioxidant defense system and hemato-biochemical indices of Cd-exposed fish. Compared with the Cd-only group, dietary supplementation with *L. plantarum* CCFM8610 restored the structure and composition of the fish gut microbiota, although the recovery was not very significant. The abundances of *Flavobacterium* and *Pseudomonas* were decreased in the Cd-plus-CCFM8610 group, indicating the potential protection of *L. plantarum* CCFM8610 treatment against Cd-induced infectious diseases. On the other hand, CCFM8610 supplementation increased the abundance of *Cellvibrio* and *Acinetobacter* in the Cd-exposed fish. Strains of *Cellvibrio* sp. have been reported to possess a specific protein (Cdae-1), which enhanced Cd accumulation when expressed in *Escherichia coli* (Mori et al. 2016). *Acinetobacter* strains such as *Acinetobacter calcoaceticus*, *Acinetobacter calcoaceticus var. antratus* and *Acinetobacter johnsonii* have been reported to have a good Cd binding ability and can be used for bioremediation of Cd-contaminated wastewaters (Minz et al. 1996; Boswell et al. 1998). Therefore, the increased abundance of these bacteria may enhance the Cd sequestration in the gut, due to cellular accumulation and bio-removal mechanisms. This may also explain the higher Cd level in the gut of the Cd-plus-CCFM8610 group than that of the Cd-only group.

Consistent with previous reports (Almeida et al. 2002; Valavanidis et al. 2006), our results demonstrated that Cd exposure inhibited the activities of antioxidant enzymes and increased the level of MDA (an indicator of the lipid peroxidation process) in the liver and brain of Nile tilapia (Figures S4 and S5). The protective effect of dietary probiotic supplementation against oxidative stress may be caused by the reduction in the tissue Cd burden and the protection of the antioxidant defense systems by *L. plantarum*

Table 4 Effects of dietary *L. plantarum* CCFM8610 supplementation on metal levels in the muscles of Nile tilapia

Treatment (group)	Mean concentration ($\mu\text{g g}^{-1}$ of wet tissue) \pm SEM				
	Cd	Ca	Fe	Mg	Zn
Control	–	204.44 \pm 24.67 ^a	2.94 \pm 0.15 ^a	260.00 \pm 4.71 ^a	3.54 \pm 0.093 ^a
8610 only	–	195.56 \pm 26.67 ^a	3.43 \pm 0.26 ^a	262.22 \pm 9.54 ^a	4.29 \pm 0.17 ^b
Cd only	0.14 \pm 0.020 ^a	364.44 \pm 43.14 ^b	3.68 \pm 0.45 ^a	265.56 \pm 6.26 ^a	5.62 \pm 0.42 ^c
Cd + 8610	0.057 \pm 0.006 ^b	378.89 \pm 46.68 ^b	3.73 \pm 0.29 ^a	263.57 \pm 9.30 ^a	5.29 \pm 0.28 ^{bc}

–, Cd concentration in the muscles was too low to detect. Data are expressed as the mean \pm SEM for each group. The superscript letters indicate statistically significant differences at a *P* value of <0.05 within each row comparison

**Fig. 4** Cycle of Cd in the aquatic ecosystem and the potential protective pathways of probiotics against tissue Cd accumulation in Nile tilapia

CCFM8610 (Zhai et al. 2014). As a downstream effect of the above-mentioned protection, the Cd-induced growth inhibition and abnormal mortality of fish were also markedly reversed by *L. plantarum* CCFM8610 treatment (Table 1).

Cd exposure has been reported to affect iron metabolism and result in microcytic hypochromic anemia, with decreased levels of hematological parameters such as MCV (Pratap 2008; Reynders et al. 2006). With the exception of MCH and MCHC, Cd exposure significantly inhibited these parameters (Figures S1 and S2), which is consistent with a previous report (Ruparelia et al. 1990). Dietary

supplementation with *L. plantarum* CCFM8610 was effective in recovering the HGB and WBC levels in the Cd-exposed fish, indicating that this strain is protective against Cd-induced dysfunctions of the immune system and protects the health status of Nile tilapia (Davis et al. 2008; Houston 1997). The restoration of ALT and AST in fish by dietary *L. plantarum* CCFM8610 (Figure S3) indicated the protective effect of this strain against Cd-induced organ dysfunctions such as liver and heart damage in Nile tilapia (Shahsavani et al. 2010). The alterations in TC, TP, TG and HDL of Cd-exposed fish demonstrated that Cd exposure also adversely affects the

lipid and protein metabolism (Ma et al. 2015). *L. plantarum* CCFM8610 treatment markedly reversed the alteration of the level of TG, which may be another reason for the enhancement of growth performance (Table 1) by the strain.

Besides the protective effects of dietary *L. plantarum* CCFM8610 supplementation against waterborne Cd exposure, its safety of use in Nile tilapia was also evaluated. *L. plantarum* CCFM8610 treatment by itself did not exert adverse effects on the growth performance, hemato-biochemical biomarkers, oxidative stress status or intestinal microbiota of the fish. Compared with the control group, the growth rate and feed utilisation were superior in the probiotic-supplied group, which is consistent with a previous report demonstrating that probiotics can enhance the growth performance of fish (Ridha and Azad 2016). *L. plantarum* CCFM8610 supplementation did not cause losses of the essential metals Ca, Fe, Mg or Zn from the muscles of fish (Table 4). Taking these analyses into consideration, it can be concluded that dietary supplementation of *L. plantarum* CCFM8610 is safe for Nile tilapia.

In conclusion, the protective effects of dietary probiotic supplementation on the growth performance, tissue oxidative stress status and Cd levels, hemato-biochemical parameters and intestinal microbiota of Nile tilapia exposed to waterborne Cd were evaluated in the present study. The results suggest that *L. plantarum* CCFM8610 can be used as a safe dietary supplement for the prevention of Cd-exposure-induced problems in aquaculture and food safety.

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

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