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Effect on Serum Parameters and Immune Responses of *Carassius auratus gibelio* Exposed to Dietary Lead and *Bacillus subtilis*

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Abstract
Lead (Pb), a heavy metal and an environmental stressor, may affect many physiological processes, including the serum index and the immune response. The aim of this study was to explore the toxic effects of Pb on the serum index and the immune response of *Carassius auratus gibelio* (*C. gibelio*) fed 0, 120, or 240 mg/kg Pb, and 10^9 cfu/g *Bacillus subtilis* (*B. subtilis*). After 15 and 30 days of dietary exposure, the serum indices and the immune responses of the fish were assessed. Dietary Pb exposure significantly affected various components of the serum index, including calcium, magnesium, glucose, cholesterol, total protein, glutamic-pyruvic transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). However, sIgA activity in the gut increased significantly following *B. subtilis* supplementation. Notable changes were also observed in the expression levels of immune-related genes, including HSP70, IgM, HSP90, IL-1β, IL-6, and TNF-α. *B. subtilis* supplementation effectively attenuated the effects of dietary Pb exposure.

Keywords *Bacillus subtilis* · Lead · Serum index · Immune response

Introduction

Lead (Pb) is a toxic heavy metal that causes adverse health effects in humans and animals. Pb is ubiquitous in the environment, occurring extensively in air, water, and soil [1]. Many studies have reported high levels of environmental Pb, even some exceeding 90 mg/kg in marine sediments [2]. At low aquatic Pb concentrations, aquatic animals, including the common carp and *Carassius gibelio*, are more likely to accumulate high levels of Pb in their tissues [3]. Humans are thus at risk of Pb exposure through consumption of aquatic products.

As a non-essential biological element, Pb is widely toxic, affecting the hematopoietic system and the immune response, causing oxidative stress, even leading to death (at high concentrations) [4–6]. In fish, hematological parameters are sensitive and reliable indicators of the physiological stress associated with heavy metal exposure, as fish blood directly interfaces with the external environment [7]. Serum parameters have also been used as pathophysiological indicators of the structural and functional status of fish exposed to toxins [6]. Several studies have suggested that heavy metals damage the blood system, causing hematocytopenia, reducing serum protein levels, and changing ion polarity [8, 9]. Pb may also suppress the immune system by inhibiting non-special immune protein activity and immune-related gene expression [4, 10].

It has recently been shown that dietary supplements protect against heavy metal toxicity [11]. Probiotics are living microorganisms that are beneficial to the host [12]. Of the various available dietary supplements (e.g., essential metals, vitamins, edible plants, phytochemicals, and probiotics) [13–16], probiotics may potentially reduce Pb toxicity [17]. For instance, it has been suggested that *Lactobacillus plantarum* alleviates heavy metal toxicity (e.g., Pb and Cd) [16, 18]. However, Lee et al. (2016) suggested that *Bacillus subtilis* might be a more effective probiotic than *L. plantarum* for aquacultural applications. *B. subtilis*, a genus of bacillus, is gram-positive and widely used as a probiotic in aquaculture because it is able to tolerate a wide range of temperatures and low pH [19].

Previously, we found that in *C. gibelio* exposed to Pb, *B. subtilis* reduced Pb accumulation in the organs, affected

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hematological parameters, and influenced antioxidant activity [4, 10, 20]. However, few studies have investigated the effects of dietary Pb and *B. subtilis* on *C. gibelio* serum parameters and immune responses. To address this knowledge gap, we herein analyzed the effects of Pb exposure and *B. subtilis* administration on the serum index, non-special immune protein activity, and immune-related gene expression in *C. gibelio*. We found that *B. subtilis* alleviated Pb toxicity in *C. gibelio*.

Materials and Methods

Fish and Bacteria

C. gibelio (62.51 ± 0.42 g) were obtained from a specialized aquatic fry farm (Jilin province, China). Fish were maintained in 80-L tanks and fed regularly with artificial feed for 2 weeks before the experiment was started. During the acclimation period, fish were fed a Pb-free diet twice daily while constantly maintaining experimental conditions at all times (dissolved oxygen, 5.91 ± 0.21 mg/L; pH, 7.4 ± 0.2; ammonia, less than 0.5 mg/L; nitrites, less than 0.05 mg/L; and temperature, 23 ± 2 °C).

B. subtilis strain was isolated from *C. gibelio* intestine; the method described by Veras et al. [21]. The bacterial strain was cultivated in Luria-Bertani (LB) medium and grown under aerobic condition at 30 °C with shaking (at 180 rpm) for 12 h.

Diet Preparation

Commercial feed (crude protein 37.7%, crude lipid 7.4%, and ash 10.8%) obtained from Jinyanhong Aquarium Products Co., Hangzhou, China, was used as the basal diet. The experimental diet was formulated by supplementing the basal diet with *B. subtilis* (at a final dose of 10^9 cfu/g diet) and/or lead acetate (Pb) (120 mg/kg and 240 mg/kg). The commercial feed was made of powder, and the powder was sifted through 120-μm mesh. *B. subtilis* and/or Pb at specified concentrations were mixed thoroughly in cooled conditions and then pelleted with a hand pelletizer. The concentration of *B. subtilis* in the feed was determined by spread plate technique (nutrient agar incubated at 30 °C for 24 h). The control diet was prepared by adding the same volume of sterile saline to the basal diet. The prepared diets were stored at 4 °C before use. The actual dietary Pb concentration is showed in Table 1 using atomic absorption spectrometer AA-6300 (Shimadzu, Japan).

Experiment Design

Two hundred seventy healthy fish were randomly distributed into six groups with three replications each (15 fish per replicate). Each group was kept in 80-L plastic tanks. Each group was exposed to dietary Pb and/or *B. subtilis*. The groups were divided as follows: CK group (control), CB group (*B. subtilis*, 10^9 cfu/g), LP group (120 mg/kg Pb), LPB group (120 mg/kg Pb plus *B. subtilis*, 10^9 cfu/g), HP group (240 mg/kg Pb), and HPB group (240 mg/kg Pb plus *B. subtilis*, 10^9 cfu/g). The fish were fed twice daily (9:00 and 15:00) for 30 days at a rate of 3% bodyweight/day. Lead acetate $(CH_3COO)_2$ Pb·$3H_2O$ was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). All experiments and handling of the animals were conducted according to the research protocols approved by the Institutional Animal Care and Use Committee, Jilin Agricultural University.

Table 1 Analyzed and actual Pb concentration (mg/kg)

Groups	Analyzed Pb concentration	Actual Pb concentration in the experiment days	
		15	30
CK	0	2.52 ± 0.51	2.95 ± 0.48
CB	0	2.34 ± 0.34	2.13 ± 0.37
LP	120	121.13 ± 1.24	119.47 ± 1.62
LPB	120	118.46 ± 1.38	118.73 ± 1.54
HP	240	238.48 ± 1.62	239.69 ± 1.36
HPB	240	241.84 ± 1.78	242.14 ± 1.98

Serum, Intestine, and Spleen Samples

At the end of the feeding trial, fish were fasted were 24 h. All fish were anesthetized and then euthanized using 300 mg/L methane-sulfonate-222 (MS-222). Blood samples were drawn from the caudal veins of all fish with sterile saline and stored at 4 °C for 24 h. The guts and spleens were removed, frozen in liquid nitrogen, and stored at − 80 °C.

Serum Assay

Plasma was separated from each serum sample by centrifugation at 4000*g* for 5 min at 4 °C. Levels of the biochemical components of the plasma (calcium, magnesium, glucose, cholesterol, total protein, glutamic-pyruvic transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), immunoglobulin M (IgM), and lysozyme (LZM)) were measured using clinical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

Determination of Secretory-IgA (sIgA) in the Gut

The concentration of sIgA in each intestinal mucosa sample was measured using a kit (Shanghai Langdon Biotechnology Co. Ltd.), following the method described by Wang et al. [22].

Total RNA Extraction and cDNA Synthesis

The frozen spleen samples were homogenized in Trizol reagent (Takara, Dalian, China), and total RNA was extracted from each sample following the manufacturer's instruction. Each dried RNA pellet was dissolved in RNase/DNase free water. Aliquot were stored at − 80 °C. RNA quality was determined using 1% agarose gel electrophoresis, and RNA quantity was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was incubated with DNase I (amplification grade; Fermentas) to remove any genomic DNA, and then reverse transcribed into cDNA using a reverse transcriptase M-MLV kit (Takara, Dalian, China). cDNA was stored at − 20 °C.

Relative Expression of Immune-Related Genes

Gene expression was analyzed in the spleen sample after 30 days of treatment. Real-time quantitative reverse transcriptase PCR (RT-qPCR) was performed using 2 × SYBR premix *Ex Taq* (Takara, Dalian, China). The primers used to amplify the immune genes were designed using Oligo 7.0 (primer sequences are given in Table 2). All RT-qPCR reactions were performed on an ABI 7500 Fast Real-time PCR system (Applied Biosystems) a minimum of three times. PCR reaction mixtures contained 10 μL of 1 × SYBR *premix Ex Taq*, 200 nM (1 μL) of each primer, 5 μL of 20× diluted cDNA, and nuclease free water to make a final volume of 25 μL. The reaction conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. The qRT-PCR data were converted into Ct values. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to *β-actin* expression.

Statistical Analysis

Statistical analyses were conducted using SPSS 20.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± standard deviation (S.D.) for each group. The entire experiment was repeated three times. Significant differences among groups were identified using one-way analyses of variance (ANOVAs), followed by Tukey's multiple comparison tests. We considered $P < 0.05$ statistically significant.

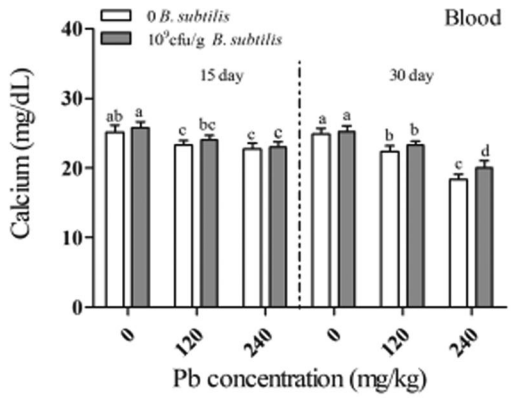
Results

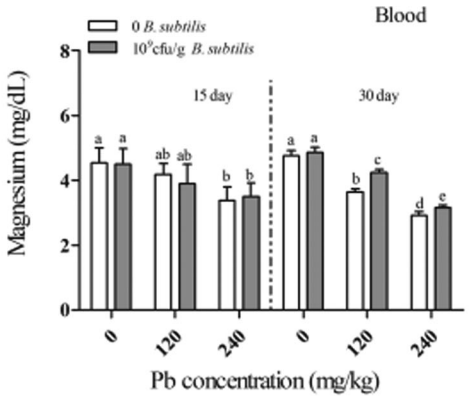
Serum Index

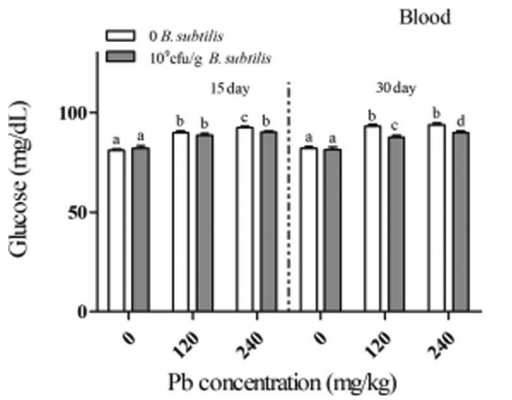
After 15 days of treatment, serum cholesterol and AST levels were significantly lower in the groups treated with 0 or 120 mg/kg Pb-*B. subtilis*, as compared to the Pb-only treatment groups ($P < 0.05$; Fig. 1). Serum glucose levels in the 240 mg/kg Pb-*B. subtilis* treatment groups were also significantly lower than in the Pb-only groups ($P < 0.05$), as was serum ALP in the 120 mg/kg Pb-*B. subtilis* treatment group ($P < 0.05$). After 30 days, serum glucose and ALT levels were significantly lower in the 120 and 240 mg/kg Pb-*B. subtilis* treatment groups as compared with the Pb-only treatment groups ($P < 0.05$), while serum magnesium was significantly higher ($P < 0.05$). Compared to the Pb-only treatment group, serum calcium was significantly higher in the 240 mg/kg Pb-*B. subtilis* treatment group after 30 days ($P < 0.05$), but AST, cholesterol, and ALP levels were significantly lower in the 0, 120, and 240 mg/kg Pb-*B. subtilis* treatment groups. Serum total protein was significantly lower in the 240 mg/kg Pb-*B. subtilis* treatment group than in the Pb-only group after 30 days ($P < 0.05$).

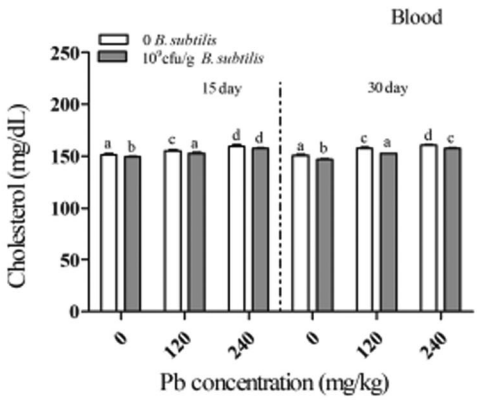
Table 2 Primers used in this study

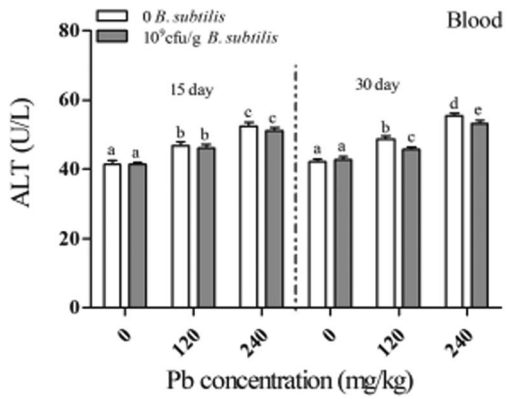
Genes	Sequences(5′-3′)		Accession no.
β-actin	Forward Reverse	TGAAGATCCTGACCGAGCGT GGAAGAAGAGGCAGCGGTTC	NM_131031.1 (Dai et al.)
HSP70	Forward Reverse	ATTGAGACCGCAGGTGGAGT GGCTGGTTGTCGGAGTAGGT	NM_131397.3 (Dai et al.)
IgM	Forward Reverse	AGCTCAACCATCTGCACCAA ATGTAAGCGAGTCCGCAGGT	GU563726.1 (Dai et al.)
HSP90	Forward Reverse	GTATGGAGCAGCAAGACCGAGAC CAACCTCAGCCTCATCTTCAGTGG	GU258544.1 (this study)
IL-1β	Forward Reverse	CAGTAAGACCAGCCTGACCTTGC GCACTCAGCGTCACAGCCTTC	AJ245635.1(This study)
IL-6	Forward Reverse	CTGCCTGTCTCAGAGATCACAAGC GCCGCAGACTATGCCGAAGAAG	AY102632.1 (this study)
TNF-α	Forward Reverse	CGCGACTGACACTGAAGACC GCAGGAGTTCTGTGGTGGTG	EU069817.1 (Dai et al.)

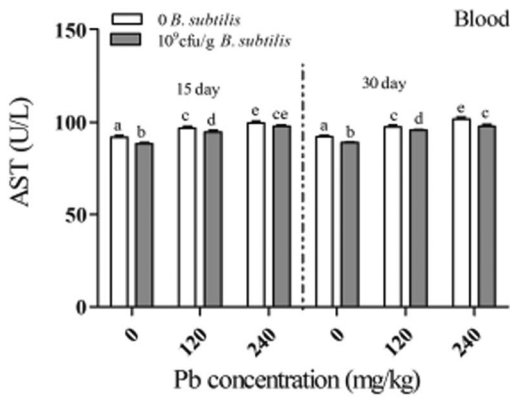

0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
Calcium (mg/dL)
Pb concentration (mg/kg)

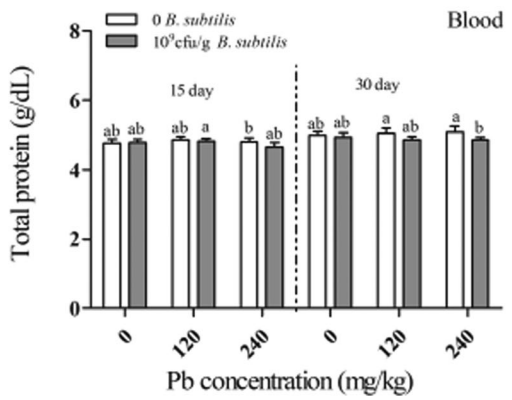

0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
Magnesium (mg/dL)
Pb concentration (mg/kg)

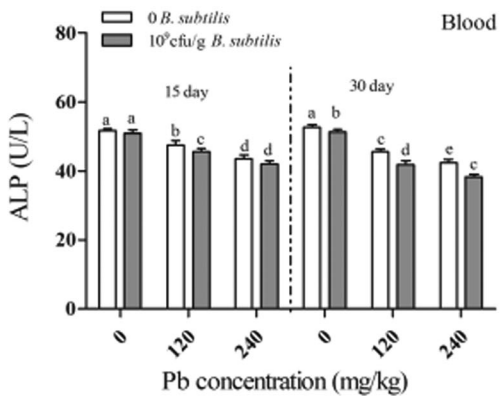

0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
Glucose (mg/dL)
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
Cholesterol (mg/dL)
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
ALT (U/L)
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
AST (U/L)
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
Total protein (g/dL)
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
Blood
15 day
30 day
ALP (U/L)
Pb concentration (mg/kg)

◀**Fig. 1** Change of serum index in *C. gibelio* ($n = 6$) exposed to the different concentration of dietary lead and *B. subtilis*. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by the Tukey test on the same sampling interval

Non-special Immune Protein Activity

After 15 days of treatment, serum IgM concentration and LZM activity level in the 0 mg/kg Pb-*B. subtilis* treatment group were significantly higher than those in the Pb-only treatment group ($P < 0.05$; Fig. 2). After 30 days of treatment, serum IgM concentration and LZM activity were significantly greater in the 0, 120, and 240 mg/kg Pb-*B. subtilis* treatment groups as compared to the Pb-only treatment group ($P < 0.05$). After 15 days, the sIgA level in the gut was significantly greater in the 0 mg/kg Pb-*B. subtilis* treatment group as compared to the Pb-only treatment group ($P < 0.05$); after 30 days, sIgA levels in the gut were significantly greater in the 0, 120, and 240 mg/kg Pb-*B. subtilis* treatment groups as compared to the Pb-only treatment group ($P < 0.05$). Compared with the Pb-only treatment group, serum LDH was significantly lower in the 0 mg/kg Pb-*B. subtilis* treatment group after 15 days ($P < 0.05$), and in the 120 and 240 mg/kg Pb-*B. subtilis* treatment groups after 30 days ($P < 0.05$).

Immune-Related Gene Expression

Compared with the Pb-only treatment group, IgM expression levels in the kidneys were significantly greater in the 0 and 240 mg/kg Pb-*B. subtilis* treatment groups ($P < 0.05$; Fig. 3). Compared with the Pb-only treatment group, the gene expression levels of HSP70, HSP90, IL-1β, IL-6, and TNF-α were significantly greater in the 120 and 240 mg/kg Pb-*B. subtilis* treatment groups ($P < 0.05$).

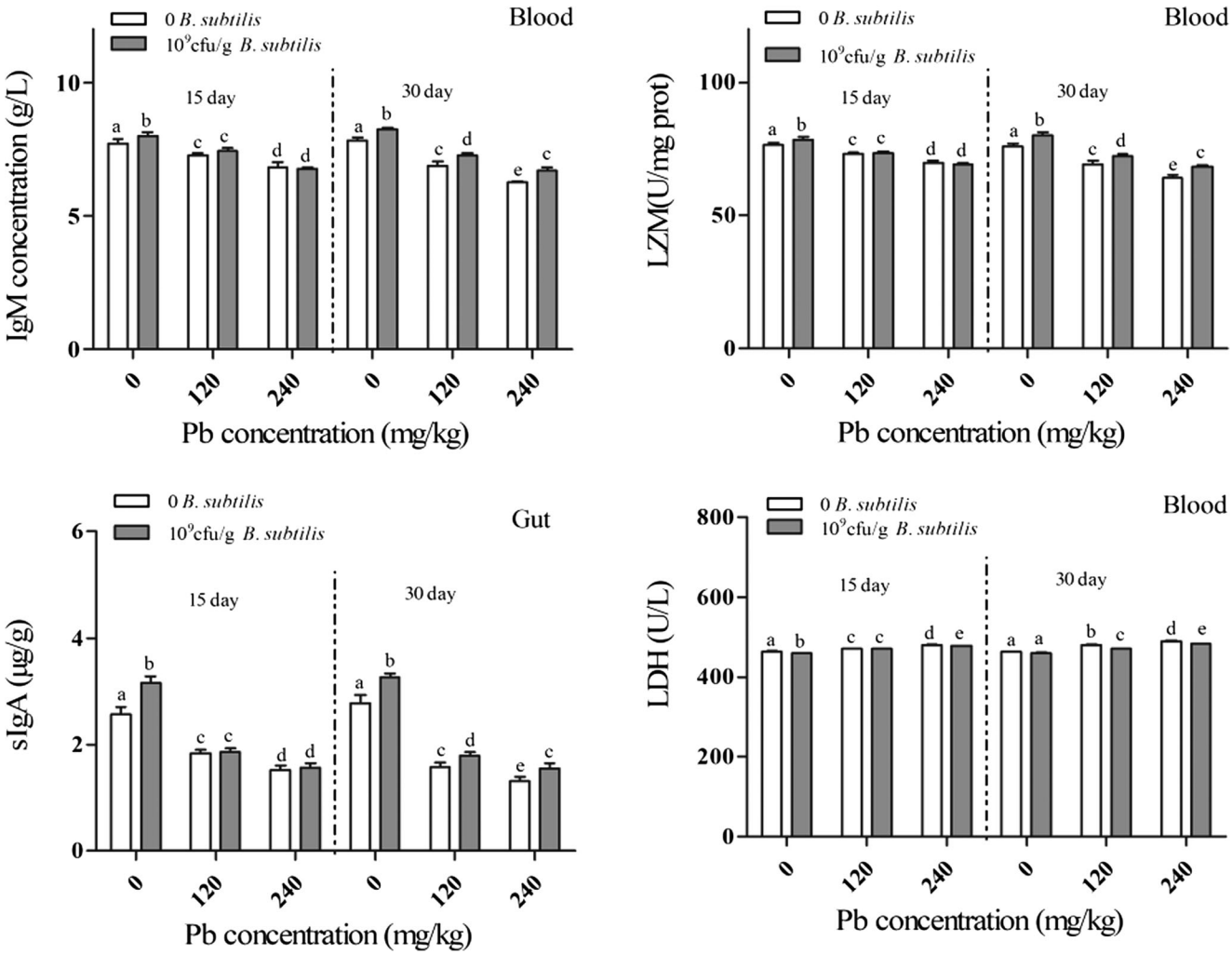
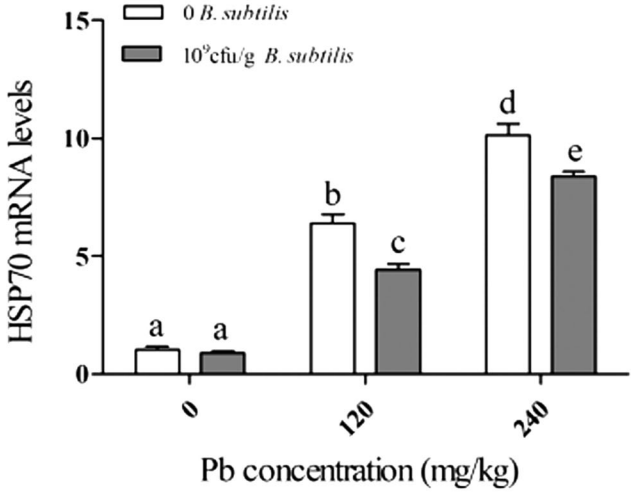
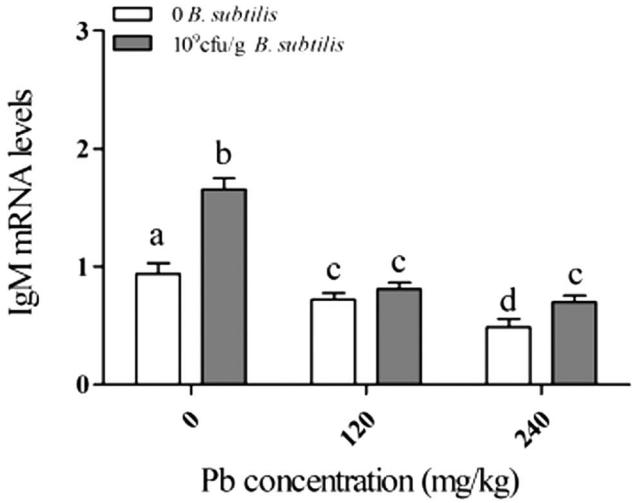
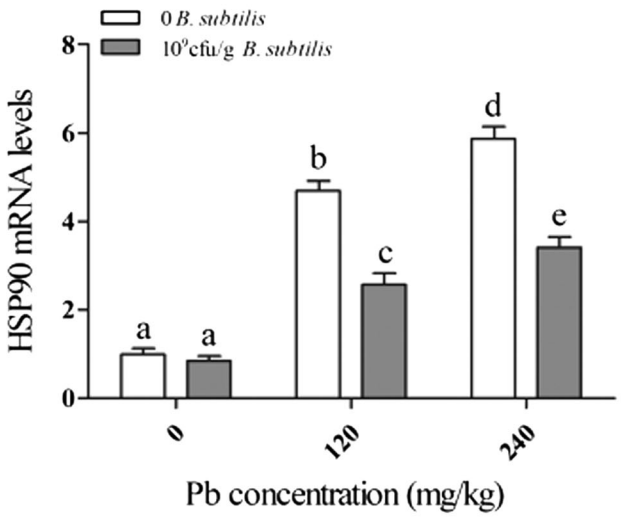
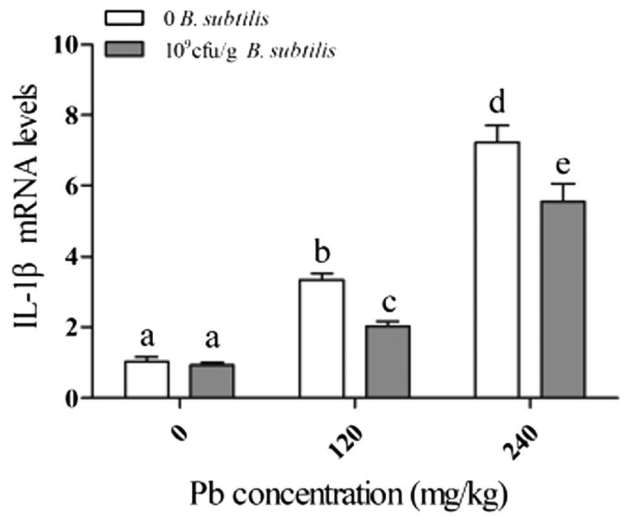


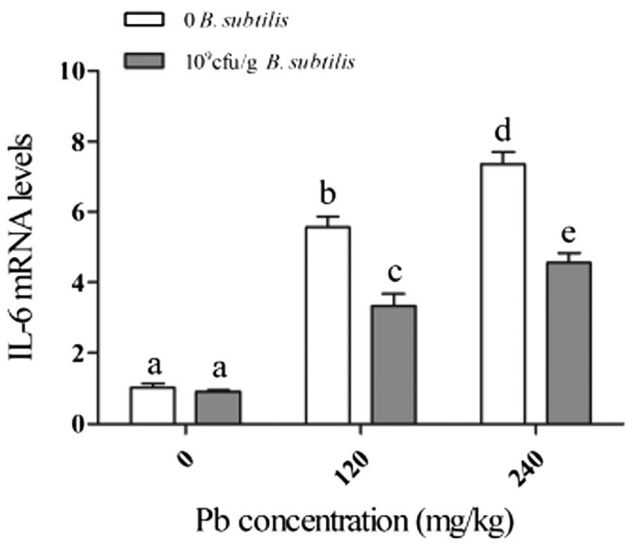
Fig. 2 Change of non-special immune protein activity in *C. gibelio* ($n = 6$) exposed to the different concentration of dietary lead and *B. subtilis*. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by the Tukey test on the same sampling interval

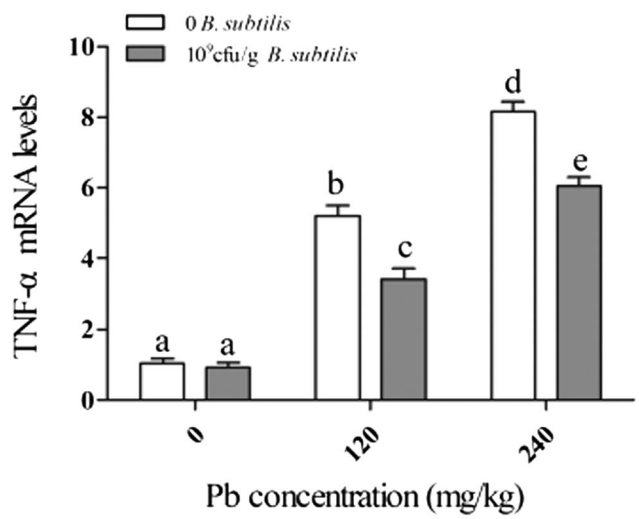

0 B. subtilis
10^9cfu/g B. subtilis
HSP70 mRNA levels
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
IgM mRNA levels
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
HSP90 mRNA levels
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
IL-1β mRNA levels
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
IL-6 mRNA levels
Pb concentration (mg/kg)


0 B. subtilis
10^9cfu/g B. subtilis
TNF-α mRNA levels
Pb concentration (mg/kg)

◀**Fig. 3** Related-immune gene expression in *C. gibelio* ($n = 6$) exposed to the different concentration of dietary lead and *B. subtilis*. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by the Tukey test on the same sampling interval

Discussion

Probiotics enhances the immunity and adjusts the serum plasma properties of the target species [23, 24]. *B. subtilis* is becoming more widely used as a probiotic for farmed aquatic species [19]. The heavy metal Pb has serious toxic effects on the hematology and immunity of aquatic organisms [4, 20]. In this study, we evaluate the effects of Pb exposure and *B. subtilis* administration on the serum index, non-special immune protein activity, and related-immune gene expression for the first time.

Hematology has been widely used to evaluate the health of animals exposed to various heavy metals, including Pb, Cd, and Sn [9, 25, 26]. Hematological changes have been reported in fish exposed to various stress-inducing substances [27, 28]. Previously, we found that fish blood noticeably accumulated heavy metals [10]. Pb accumulation in the blood can suppress protein activity and damage the plasma balance [29]. Kim et al. (2017) demonstrated that Pb exposure affected calcium and magnesium concentrations in the blood of the rockfish, *Sebastes schlegelii* [25]. Similar results were observed in the rainbow trout [30]. Rogers et al. (2004) suggested that Pb exposure affected ion regulation by significantly reducing the Ca^{2+} concentration, and Ca^{2+} ATPase activity in rainbow trout [31].

Here, although Pb exposure led to considerable decreases in blood calcium and magnesium levels in *C. gibelio*, *B. subtilis* supplementation reversed these decreases. Zhang et al. (2016) reported that tributyltin disrupted feeding and energy metabolism in goldfish [32]. Blood glucose and cholesterol levels are general secondary responses to stress in fish; thus, these levels can be considered sensitive indicators of environmental stress [33]. Pb damages the conversion of glucose to glycogen [34].

As a critical structural component of various membranes, cholesterol is a sensitive indicator of heavy metal–induced environmental stress. Firat et al. (2011) reported that cholesterol levels in Nile tilapia were significantly increased by dietary Pb exposure, possibly because liver and kidney failure led to the release of cholesterol into the blood [35]. ALT and AST, which are synthesized in the liver, are frequently used as critical indicators of liver impairment. Heydarnejad et al. (2013) reported that the levels of ALT, AST, total protein, and ALP in rainbow trout blood were significantly increased by dietary Cd [9]. Similar results were observed in rockfish [25]. Previously, we found that dietary exposure to Pb in *C. gibelio* resulted in a substantial accumulation of Pb in the blood and liver. Here, we demonstrated that the serum index of *C. gibelio* was altered by dietary Pb exposure. Probiotics and secondary metabolites from probiotics benefit the host [36, 37]. Secondary metabolites mainly include microbial exopolysaccharides (EPS) and proteins [36]. Feng et al. (2012) reported that microbial exopolysaccharides from lactic acid bacteria absorb Pb(II) [38]. Here, *B. subtilis* supplementation effectively reversed and reduced the alterations caused by dietary Pb exposure. This was probably because *B. subtilis* increased the absorption of Pb by EPS in the blood and liver. Once the concentration of lead in the tissues was reduced, the serum index recovered.

Pb inhibits immune protein activity and alters the expression of immune-related genes [4, 20]. Immune proteins in the serum and gut play important roles in non-special immune responses [26]. Dai et al. (2018) suggested that the levels of IgM and LZM in the blood of Crucian carp were significantly decreased after exposure to waterborne Pb (at 0.05, 0.5, and 1 mg/L) [4]. Wu et al. (2017) reported that Pb exposure injured the crustacean metabolic organ, inhibiting the non-special immune response [5]. Zhang et al. (2016) demonstrated that zebrafish exposed to tributyltin for up to 56 days had lower concentrations of IgM and LZM in the gut [26]. LDH may also play an important role in the response of the immune system to viral infection or heavy metal exposure.

However, dietary supplementation with *B. subtilis* may enhance the activity of immune proteins in the serum and gut [22, 26]. In the common carp, different combinations of *Bacillus* supplements enhanced IgM and LZM activity in the serum and sIgA activity in the gut [22]. Consistent with these results, immune proteins in the serum and gut decreased significantly after Pb exposure in *C. gibelio*, perhaps because Pb disrupted the structures of these proteins. However, *B. subtilis* supplementation effectively reduced the alterations caused by dietary Pb exposure. This was probably because *B. subtilis* induced the repair of protein structures by secondary metabolites. Secondary metabolites from probiotics may regulate the host immune response [39]. Several studies have demonstrated that secondary metabolites play a protective role in mammalian models of inflammatory bowel disease [40, 41]. In the common carp, the expression of immune-related genes (including HSP70, IL-1β, TNF-α, and IL-10) was improved, and disease resistance was increased due to the presence of secondary metabolites after *B. licheniformis* administration [39]. Here, the expression levels of immune-related genes (including HSP70, HSP90, IL-1β, IL-6, and TNF-α) increased significantly after Pb exposure in *C. gibelio*, while IgM significantly decreased. *B. subtilis* supplementation also effectively regulated immune-related gene expression after dietary Pb exposure. Thus, secondary metabolites may stimulate immunity. Immune regulation might be one of the main mechanisms by which *B. subtilis* alleviates Pb toxicity.

Conclusion

Our results suggested that *B. subtilis* regulates the serum index, increases the activity of immune proteins, and alters immune-related gene expression following Pb exposure in *C. gibelio*. Thus, *B. subtilis* might potentially alleviate the toxic effects of Pb exposure in aquaculture species.

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Compliance with Ethical Standards

This study was approved by the Ethics Committee of Jilin Agricultural University with ID no. 20121008. All authors read this guide carefully. All subjects signed their informed consents before participation.

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

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