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Research Article

Dietary Supplementation of *Bacillus subtilis* as Probiotic affects Haemato-immunological Parameters of Common Carp (*Cyprinus carpio*)

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ABSTRACT

The current study was conducted to investigate the influence of *Bacillus subtilis* feed additive on hematological parameters, blood serum parameters and the levels of cytokines, including Transforming growth factor beta (TGF-β) and Interleukin 1 beta (IL-1β), of common carp. The probiotic was isolated from local soils using PCR approach for amplification of 16S rDNA and added to the diet in a dose of ~10^7 CFU g^{-1}. The feeding trial was carried out for six weeks. A total of 64 fish (77.2 ± 0.86 g) were randomly divided into two groups: control group (basal diet) and experimental group. Compared to the control group, the carp fed probiotic supplemented diet displayed a significant elevation in mean cell haemoglobin concentration (P <0.05). The other haematology parameters were not significantly affected. The blood serum profiles i.e., blood urea, cholesterol and random blood sugar, were not affected by the experimental diet. However, the group of fish fed probiotic supplemented diet revealed high significant in serum creatinine compared to the control group. The experimental carp displayed significant improvements in IL-1β level compared to the control group (P=0.004). On the other hand, the level of TGF-β was lower in the probiotic treated fish but no significant differences were observed (P=0.05). The results of the present study, display a possible role for *B. subtilis* for improving the health status of common carp.

Keywords: *Bacillus subtilis*; Common carp; Haematology; Serum biochemistry; Immune cytokines

INTRODUCTION

As a result of increase in aquaculture practices, the emergence of a wide range of disease causatives has resulted in infectious diseases becoming the major limitation to productivity. In aquaculture, disease outbreaks are simply treated by antibiotics and other chemical medications. The use of antibiotics has resulted in the increase of antibiotic resistant bacteria. Additionally, antibiotics in aquacultureal products may be harmful to human health by inhibiting or killing the normal microbiota of the gastrointestinal tract [1]. Thus, the use of probiotics as an eco-friendly approach has been found to enhance the physiology, growth efficiency, and immune reactions of aquaculture-related species.

In aquaculture, "a probiotic organism can be regarded as a live, dead or component of a microbial cell, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed *utilization*, stress response or general vigor, which is achieved at least in part via improving the host's microbial balance or the microbial balance of the ambient environment" [2].

Probiotics action may depend on the probiotic itself, the dosage applied, treatment period, and route and frequency of administration [3]. Indeed, for almost 50 years, *Bacillus* species have been used as probiotics. The species most often used include *B. subtilis* and *B. licheniformis*. These bacteria, when used as probiotics in the form of a feed supplement, are non-pathogenic and non-toxic and can contribute to stimulations of immune system and have antimicrobial activity and antagonist activity [4,5]. *B. subtilis* grow effectively with low-cost sources of carbon and nitrogen because their enzymes are very effective in breaking down a wide range of animal and vegetable proteins, carbohydrates and lipids into their constituent units [6].

The haematological parameters and blood biochemistry are considered to be indicators for monitoring health condition in fish following feeding on dietary supplementation of probiotic and different stresses in fish farming [7]. Although probiotic applications have been widely used in fish and shellfish aquaculture, no information is available concerning probiotic supplementation in common carp in Iraq. Therefore, the current study was carried out to examine the effect of *B. subtilis* on some haematological,

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immunological and serum biochemical parameters of common carp.

MATERIALS AND METHODS

Experimental design

Common carp (77.2 ± 0.86 g) were obtained from a local commercial fish farm and transferred alive in polyethylene bags to the aquarium of Agriculture College, University of Kerbala (Iraq). Fish were adapted to the new conditions for 2 weeks before the commencement of the trial. Five fish were randomly distributed into each aquarium (7 aquaria of 30L) which were filled with unchlorinated water. Each diet was randomly assigned to four aquaria and a control group was fed with bacteria-free food.

The probiotic bacterium, *B. subtilis*, was grown in 50ml of MRS broth and incubated at 37°C for 18 h in a shaking water bath as described elsewhere [8]. After incubation, the bacterial cells were centrifuged (2000 × g for 5 min) and pellets obtained were washed twice with sterile phosphate-buffered saline. Pellets were resuspended in 10 ml of fish oil and A 590 nm and plate counts of probiotics after 24-h incubation at 37°C were measured in order to standardize the number of bacteria (10^7 CFU ml^{-1}). The required amount of bacterial suspensions was top-dressed onto basal diets (ALLER AQUA; 30% crude protein, 7% crude lipid and 6.1% fiber) slowly, mixing part by part in a drum mixer to achieve a dose of ~ 10^7 CFU g^{-1}. Control diets contained the same volume of fish oil without bacteria. The fish were handfed with treatment or control diets at a rate of 1.5-2% of the body weight twice per day (07:00 and 17:00) for a period of 6 weeks.

Identification of B. subtilis (MN756672)

Phenotypic identification of a representative selection of isolates of *B. subtilis* was conducted by using *16S rRNA* gene sequencing as described below.

Purification of bacterial DNA: One ml of an overnight culture was centrifuged then subjected to DNA extraction by using the G-spin™ Genomic DNA Extraction Kit, iNtRON Co., Seoul, Korea. The DNA was stored at -20°C until used.

Amplification of the 16S rRNA gene: The PCR for *16S rRNA* gene was carried out with universal primers: the forward primer 27F (AGAGTTTGATCMTGGCTCAG) and the reverse primer 1491R (GGTTACCTTGTTACGACTT) [9].

PCR cycling was carried out by using a Gene Amp PCR System 080725 apparatus (Multigene Labnet International Inc., USA) under conditions as described elsewhere [10]. To compare with the nearest known alignment identities for the partial *16S rRNA* sequences, Sequence results were then submitted to a BLAST search in GenBank (http:/ blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure 1).

Haematological parameters

Blood collection: Following the feeding trial, 7 fish from each group (treatment and control) were randomly collected. Fish were sedated by immersion in water containing clove oil (25 mL per 1 L). Blood samples were taken from the caudal vein using a 27-gauge needle and divided into two parts. The first part was transferred to tubes rinsed in heparin (15-units per mL) to be used for haematological parameters including haematocrit, haemoglobin, erythrocyte

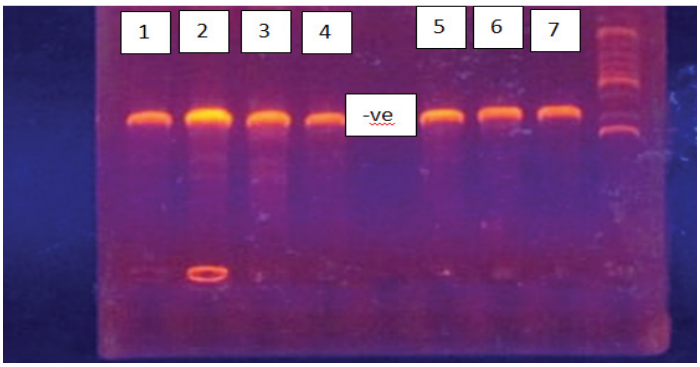

Figure 1: Agrose gel electrophoresis of PCR products from different soil location, 1-*Bacillus haynesii* (MK9516861), 2- *Bacillus spp.* (KC9545542), 3-*Acetobacter indonesiensis* (MN187257), 4-*Bacillus velezensis* (MN737993), 5-*Bacillus subtilis* (MN756672), 6-*Bacillus subtilis* (MN756672), 7-*Bacillus atrophaeus* (MN756646).

counts (RBC), total leucocyte counts (WBC), differential leucocyte counts, which were measured with a haematology analyzer (Swelab Alfa, Germany). The remaining blood-samples were allowed to clot at room temperature for 3 h. Further, tubes were centrifuged at 2000 × g for 5 min and the serum was collected and stored at -20°C until use to determine the levels of blood urea, serum creatinine, cholesterol and random blood sugar (RBS) which were measured by an Autoanalyser (Swelab Alfa, Germany).

Haematological indices: Using the formulas mentioned in Klontz et al. [11], mean cell hemoglobin concentration (MCHC), mean cell hemoglobin (MCH) and mean cell volume (MCV) were determined.

MCHC (g/dl)=Hb/Hct × 100

MCH (pg)=Hb/RBC × 10

MCV (fl)=Hct/RBC × 10

Cytokine levels assay

The levels of TGF-β and IL-1β in common carp sera were determined by an ELISA assay using Elba science® Kits following the manufacturer's instructions. In brief, 50 µL of standard solution which contained biotinylated antibody was added to wells. Forty µL of samples and 10 µL of anti-TGF β and 10 µL of anti-IL-1β antibodies were added separately to the sample wells. After addition of 50 µL of streptavidin-HRP, the solution was well mixed and then the wells were sealed with sealer and incubated at 37°C for 60 min. The sealer was removed and the wells were then washed 5 times with wash buffer. The wells were covered with new sealer and incubated for 10 min at 37°C in the dark, after addition of 50 µL of substrate solutions A and B to each well. Reaction was stopped by adding 50 µL of stop solution to each well and the blue color changed to yellow. Finally, the optical density (OD) was determined with a microplate reader (ELX 800, Biotek, USA) at 450 nm. All measurements were conducted in triplicate.

STATISTICAL ANALYSIS

All data are demonstrated as mean ± SD of the replicates. To assess the haemato-immunological differences between experimental and control groups, an independent two-sample t-test was used. All statistics were conducted using version 17 (IBM, Pennsylvania, USA) of Minitab statistical software. The accepted levels of significance in all cases were $P<0.05$.

RESULTS

Haematology parameters

The haematological parameters of common carp fed the *B. subtilis* and control diets are presented in Table 1. RBC, MCV, MCH and Hb levels were not significantly ($P > 0.05$) affected by the dietary probiotic in the present study. However, MCHC and HTC were significantly increased in the probiotic-fed fish compared to the control group ($P<0.05$). The WBC count showed a clear increase in the probiotic-fed fish that was not quite significant ($P=0.059$).

Serum biochemistry profiles

Serum biochemistry parameters of common carp fed probiotic and control diets are summarized in Table 2. Blood urea remained unaffected by the probiotic diet. The present findings revealed a lower significant value of serum creatinine in the fish fed with probiotic than that of the control group of fish. In addition, no significant differences were observed, in spite of higher serum cholesterol and random blood sugar levels were observed in fish fed dietary *B. subtilis* compared to the control group.

Interleukins

The levels of IL1 β in the serum of common carp fed treatment and control diets are showed in Figure 2. The serum of probiotic-fed fish displayed significant increase of IL-1β ($P<0.004$) compared to the control treatment. In contrast, the anti-inflammatory cytokine genes, TGF-β level in the serum of common carp decreased following the probiotic administration but no significant differences were observed ($P=0.05$) (Figure 3).

DISCUSSION

The haematological parameters and blood biochemistry are deemed to be indicators for monitoring health condition in fish following feeding on diet supplementation of probiotic and different stresses in fish farming [7]. In the present study, haematocrit, MCV and MCHC display high significant values in the probiotic-fed fish group in comparison to the control group. Several studies have reported variable haematology parameters obtained after administration of *B. subtilis* in different species of fish. For example, RBC, haemoglobin content and WBC were decreased and improved, respectively in Indian major carp *Labeo rohita* fed diet supplemented with *B. subtilis* [12]. A study of Kamgar et al. [13] reported no improvement of haematology parameters in rainbow trout feeding on *B. subtilis* feed additive. Groups of common carp had improvement levels in HCT, RBCs, WBCs, and Hb when fed different bacterial strains as probiotics supplemented diets [7,14]. The reason behind these inconsistent results is not clear but it could be because of differences in probiotic species and levels, administration time and variability in fish species. The results mentioned above suggest that *B. subtilis* leads to increase of the percent of WBCs and lymphocytes, which can stimulate the immune system in fish.

It is worth noting that serum alanine aminotransferase (ALT), creatinine and urea are considered essential criteria for the evaluation of unusual feedstuffs and new feed additives for the stage appropriate for addition. The findings obtained from the current study revealed that blood urea, serum creatinine (marker of kidney function), random blood sugar (RBS) and blood cholesterol were not affected by adding *B. subtilis* to the feed of common carp for 6 weeks. In line with present results, Sharifuzzaman et al. [15] demonstrated that serum haemoglobin, urea, creatinine and blood glucose of juvenile rainbow trout were not affected by feed supplemented with a ~ 10^7 cells g^{-1} *Rhodococcus* or ~ 10^8 cells g^{-1} *Kocuria* preparation.

Table 1: Haematological parameters of common carp fed the control diet or the control diet supplemented with the probiotic *Bacillus subtilis*.

Blood parameters	Control	Probiotics	P -value
WBC (× 10^3 mm^3)	35.4 ± 1.1^a	49.7 ± 8.3^a	0.059
Lymphocytes (%)	91.1 ± 9.7^a	95.8 ± 1.5^a	0.54
Granulocytes (%)	0.35 ± 0.2^a	1.2 ± 0.5^a	0.129
MID (%)	1.75 ± 0.9^a	3.05 ± 0.9^a	0.448
Erythrocyte count (× 10^6 mm^3)	0.30 ± 0.2^a	0.25 ± 0.05^a	0.128
Hb (g dL^{-1})	9.6 ± 0.6^a	10.1 ± 1.5^a	0.136
Hematocrit (HCT)	4.5 ± 0.9^a	7.4 ± 1.4^b	0.030
MCV (fL $cell^{-1}$)	204.2 ± 1.4^a	216.6 ± 7.8^b	0.027
MCH (pg $cell^{-1}$)	463.6 ± 1.4^a	350.9 ± 60.5^a	0.32
MCHC (g dL^{-1})	161.3 ± 22.8^a	214.4 ± 10.0^b	0.024

Means with the same letter in each row are not significantly different ($P > 0.05$). The data are expressed as the means ± S.D (n=7).

MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, MID: Monocytes, Eosinophils, Basophils and Blasts

Table 2: Serum biochemistry parameters of common carp fed with probiotic-supplemented or control diets for 6 weeks.

Biochemistry parameters mg dL^{-1}	Control	Probiotics
Blood urea	26 .2 ± 1.8^a	26.0 ± 3.9^a
Serum creatinine	0.8 ± 0.4^a	1.3 ± 0.6^b
Random blood sugar (RBS)	181.3 ± 62.4^a	188.5 ± 27.2^a
Blood cholesterol	124.0 ± 24.1^a	130.0 ± 13.6^a

Means with the same letter in each row are not significantly different ($P > 0.05$). The data are expressed as the means ± S.D (n=7).

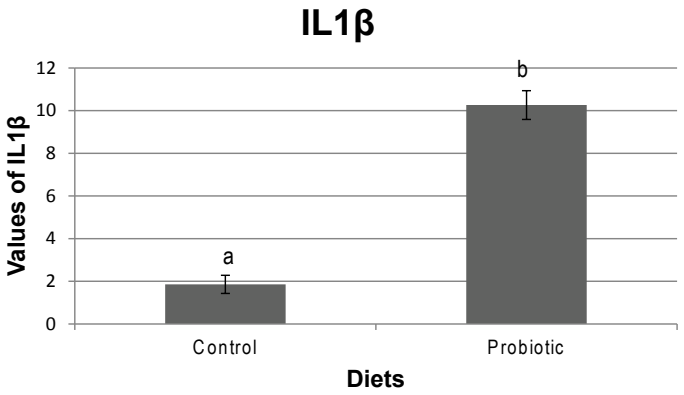

Figure 2: Serum level of IL-1β in common carp fed treatment and control diets. (Bars with the different letters between treatments are significantly different (P<0.05). Values are mean ± SD represented by vertical bars *of 7 replicates).*

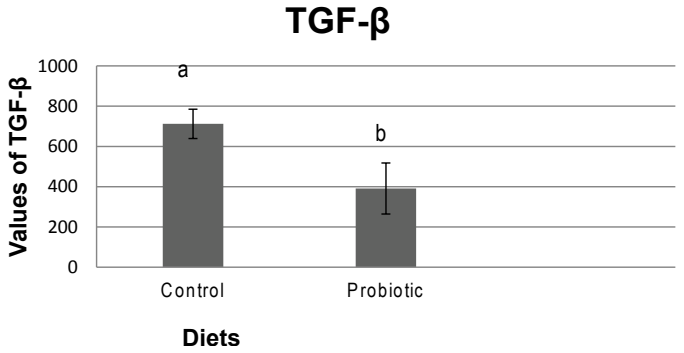

Figure 3: Serum level of TGF-β in common carp fed treatment and control diets. (Bars with the different letters between treatments are significantly different (P<0.05). Values are mean ± SD represented by vertical bars *of 7 replicates.)*

In contrast to the currents findings, Panigrahi et al. demonstrated that the commercial probiotics containing *Lactobacillus rhamnosus significantly increased the plasma cholesterol* of rainbow trout. Similarly, Al-Dohail et al. [16] reported that total serum protein, Ca, Mg, Cl, blood glucose and cholesterol were significantly enhanced by the supplementation of *Lactobacillus acidophilus* in African Catfish diets. On the other hand, Dawood et al. [17] reported that *Lactobacillus rhamnosus* or/and *Lactococcus lactis* can decrease the plasma total cholesterol and triglyceride levels of red sea bream. Species, diets fed or other environmental factors could be the reasons for the slight differences in above mentioned findings.

Panigrahi et al. stated that indigestible food-derived carbohydrate can be fermented by probiotic bacteria to generate short chain fatty acids in the digestive tract which in turn inhibit production of hepatic cholesterol and/or redistribute cholesterol from plasma to the liver by decreasing the systemic levels of blood lipids. Moreover, some bacteria can decrease the cholesterol absorption from the gut with resulting effects on cholesterol metabolism. Decreased levels of cholesterol indicate possible illness, increased levels of physiological discomfort (stress), and lipid metabolism dysfunction. The results obtained in the current study indicate that dietary probiotics had no adverse effects on the kidney and liver functions.

Pro-inflammatory cytokine IL-1β is primarily created from monocytes and macrophages, considered as biomarkers for testing immune regulation by activating lymphocytes or by inducing other cytokines to be released which are capable to elicit the activation of macrophages, NK cells and lymphocytes [18]. In the current study, significant elevation in the levels of IL-1β was noticed in *B. subtilis*-fed fish in comparison to the control fish. Supporting this observation, previous studies showed up-regulation in the expression of IL-1β after administration of probiotic feed additives in fish [8,19-21]. In contrast to the current results, *Labeo rohita* which were injected 0.1 mL of phosphate-buffered saline (PBS) containing a water-soluble fraction of purified biosurfactant at 200 (S200), or 300 (S300) μg mL^{-1} significantly down-regulated the levels of IL-1β [22].

TGF-β is a pleiotropic cytokine with different roles in the immune system which inhibits proliferation and differentiation of B and T cells and helps to down-regulate the expression of pro-inflammatory cytokines including IL-1β, TNF-α, and Interferon gamma (IFN-γ) [23,24]. Data obtained from the current study revealed that the levels of TGF-β in serum of common carp fed probiotics supplemented diet tended to significantly decrease compared to the control group (P=0.05).

Consistent with these findings, previous studies demonstrated that feed supplemented with different species of probiotics bacteria *[8] and prebiotic* [25,26] significantly *down-regulated the expression of anti-inflammatory cytokine.* In contrast to the present results, several studies revealed up-regulation of TGF-β in different fish species after probiotic bacterial supplementation [21-26].

The significant improvement of IL-1β in the current research indicates that probiotics supplemented diet could induce *inflammatory response* supported by elevation of WBC, and there was no excessive or detrimental inflammatory response, concomitantly with increasing the levels of TGF-β in the *B. subtilis*-fed fish compared to the control group. However, this hypothesis needs to be confirmed by histological observations of intestine tissue using light microscopy.

CONCLUSION

The addition of *B. subtilis* to commercial diet at a dose of ~ 10^7 CFU ml^{-1} stimulated immune response via increased levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines. However, further studies need to be conducted to evaluate the influence of immuno-stimulant, mechanisms of action and the most effective levels of *B. subtilis* for the activation of the immune system.

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AUTHORS CONTRIBUTIONS

Ali Al-Hisnawi contributed to design the study, analysis the data statistically and writing of draft manuscript. Doaa Beiwi contributed to prepare fish diet, feed and take care of fish during rearing period, lab work and samples collection. All data generated or analysed during this study are included in this published article.

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