Research Article

Efficacy of dietary supplementation with endogenous *Bacillus subtilis* on growth performance, immune response and gene expression of juvenile rainbow trout (*Oncorhynchus mykiss*)

M. Bashiri[1], M. Soltani[1,2*], E. Pirali Kheirabadi[3], S. S. Mirzargar[1], S. Shafiei[4], H. Momeni[5]

[1] Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Iran
[2] Fresh Water and Fish Health Group, Centre for Sustainable Aquatic Ecosystems, Harry Butler Institute, School of Veterinary and Life Science, Murdoch University, Murdoch, WA, Australia
[3]Department of Fisheries, Faculty of Natural Resources, Shahrekord University, Shahrekord Province, Iran
[4]Department of Food hygiene, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord Province, Iran
[5]Aquatic Animal Health Unit, Department of Veterinary Medicine, Iran Veterinary Oragansiation, Shahrekord Province, Iran

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Abstract

Potential of a host-associated probiotic, *Bacillus subtilis*, was successfully screened from 6 isolates from the intestines of healthy rainbow trout (*O. mykiss*) based on multiple probiotic characteristics in vitro assays, such as, hemolytic activity, biofilm formation, extracellular enzyme activity, inhibitory activity against pathogens, Physicochemical tolerance to gastrointestinal stress, cell surface hydrophobicity, autoaggregation, and antibiotic susceptibility. A total of 450 *O. mykiss* fingerlings were randomly distributed in three groups (one control and two probiotic-treated groups in triplicate) and fed with the basal (the control) and *B. subtilis*–supplemented diets (at a concentration of 5×10^7 and 10^8 cell/mL *B. subtilis*, over a period of 60 days. After eight weeks of feeding trial revealed that dietary supplementation of *B. subtilis* 6 at all two concentrations (5×10^7 and 10^8 cell/mL of *B. subtilis*) immunological responses, biochemical parameters, antioxidant parameter and immune-relevant gene expression (interleukin 1β (*IL-1β*), *HSP70* and *TGF-β*))) relevant to immunity were analyzed on the 30th, and 60th day post-feeding. After the 60-day feeding period, a significant (p<0.05) enhancement in some immune-biochemical response and immune gene expression was evident. Serum AST, ALT levels exhibited a significant (p<0.05) decrease. The inclusion of *B. subtilis* in the diets led to a substantial (p<0.05) increase in the survival of *A. hydrophila*–challenged *O. mykiss*, thereby highlighting the potential of *B. subtilis* as a beneficial probiotic for aquaculture in short-time period.

Keywords: Probiotic, *Bacillus subtilis*, Rainbow trout, Disease resistance

***Corresponding author's emial:** msoltani@ut.ac.ir

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Introduction

Aquaculture is the fastest-growing sector, with an average consumption of 50% of the total fish ingested per capita, due to the growing demand for high-quality protein (FAO, 2021). Aquaculture is anticipated to emerge as the primary source of seafood globally by 2028 (OECD/FAO, 2019). Nevertheless, the long-term stress due to the aquaculture intensification makes the animal more susceptible to pathogenic infections (Mohapatra *et al*., 2013).

The primary issue with this lucrative industry, particularly in an intensive culture, is the occurrence of diseases (Mohammadian *et al*., 2018; Buyukdeveci *et al*., 2023).

The use of antibiotics is a common practice in aquaculture; however, it generates a selective pressure for drug-resistant bacteria that may be introduced into the food chain from fish to humans (Banerjee and Ray, 2017b). The accumulation of antibiotics in the tissue and antibiotic-resistant bacteria, as well as the immune-suppression of fish, pose significant threats to public health and the environment (Lin *et al*., 2017; Nayak, 2021; Golas and Potorski, 2022; Docando *et al*., 2022b). Public health organizations are implementing restrictions on antibiotic usage and advocating for the adoption of sustainable preventative methods including immunostimulants, vaccines, probiotics, prebiotics, and medicinal herbs in the aquaculture sector to address the antimicrobial resistance among fish infections (Reverter *et al*., 2020). Probiotics are an alternative approach to prevent and control diseases in aquaculture as they can be used as therapeutic, prophylactic, and growth supplements (Pérez-Sánchez *et al*., 2014; Lin *et al*., 2017; Assefa and Abunna, 2018; El-Saadony *et al*., 2021). However, the majority of commercial probiotics currently used in aquaculture are not sourced from aquatic animals or the aquatic environment. Thus, in recent years, attempts have been focused on host-associated probiotics and their use in aquaculture. Zhang *et al*. (2024) discussed the safety concerns associated with utilizing probiotics from different species in aquaculture. They proposed the development of probiotic strains that are tailored to each species, instead of using probiotic strains isolated from one species for others (Zhang *et al*., 2024).

Unfortunately, non-indogenous probiotics are often incapable of the well-established local microbiome over

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a prolonged period. Therefore, they need to be continuously administered during the fish culture process (Casula and Cutting, 2002; Giatsis *et al*., 2016). Therefore, the establishment of probiotics in animal gastrointestinal tract is a crucial factor to take into account when choosing probiotics (Pérez-Sánchez *et al*., 2014).

The gut-associated microbiota is influenced by various microorganisms present in the rearing water and feed, and the diversity equilibrium of beneficial and pathogenic bacteria will be influenced by the type of culture system (Wang *et al*., 2020). This is due to the fact that host-associated probiotic microorganisms are more adapted to environmental changes in the host gastrointestinal tract, which enables them to provide the anticipated beneficial effects (Tarkhani *et al*., 2020; Van Doan *et al*., 2020; Moustafa *et al*., 2021).

The intestinal tract of fish contains a variety of bacteria, such as *Pseudomonas spp*., *Aeromonous spp*., *Enterobacteriaceae spp*., *Micrococcus* spp., *Escherichia spp*., and *Bacillus spp*. that are crucially important for fish health status (Rajan *et al*, 2024). *Bacillus spp*. are well-known for their probiotic potential and ability to produce natural antimicrobial compounds that can combat bacterial pathogens and they are particularly abundant in the guts of fish (Docando *et al*., 2022a). *Bacillus spp*. are characterized by their ability to form durable spores (Abdollahi-Arpanahi *et al*., 2019). They can influence the gastrointestinal microbiota and promote intestinal physiology (Soltani *et al*., 2019). They possess a variety of unique characters including production of various enzymes, antimicrobial peptides and metabolites that able to tolerate a wide range of physiological conditions (Liu *et al*., 2020). Utilization of certain species of *Bacillus* can increase growth performance and immunity of fish (Soltani *et al*., 2019; Ghosh *et al*., 2019; Kuebutornye *et al*., 2020a).

Among bacilli bactereia, *Bacillus subtilis* is commercially valuable microorganism due to its capacity to produce a wide range of antibiotics (Olmos and Paniagua-Michel, 2014). It has been demonstrated that administering *B. subtilis* to fish can increase animal survival rate when faced with antigens (e.g., Zhang *et al*., 2019; Zhou *et al*., 2019a), enhance growth (Soltani *et al*., 2019); improve their immune status (Nayak *et al*., 2021;

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Simon *et al*., 2021; Tachibana *et al*., 2021), modulate some aquatic animal innate immune responses (Soltani *et al*., 2019; Zhou *et al*., 2019b; Ringo *et al*., 2020), enhance the raising water quality (Soltani *et al*., 2019; Olmos *et al*., 2020) and even reduces the level stress (Abdollahi-Arpanahi *et al*., 2018; Eissa *et al*., 2018).

Rainbow trout industry is the second largest aquaculture in Iran with an annual production of about 200000 tones. The industry is suffering from various infectious diseases including bacterial diseases such as yersiniosis, streptococcosis, lactococcosis, and aeromoniasis as well as viral infections including infectious hematopoietic necrosis, infectious pancreatic necrosis and viral hemorrhagic disease. The frequent outbreaks of these infectious diseases have caused a high impact to the aquatic ecosystem and public health due to tremendous usage of various antibiotics and chemicals in the trout aquaculture sector in Iran. Therefore, alternative methods such as probiotic therapy are critically important nowadays in trout framing. However, few studies have been directed to evaluate the efficacy and potency of endogenous bacilli bacteria, particularly *Bacillus subtilis*, in the form of probiotic on growth, immunity and disease resistance of rainbow trout in Iranian trout aquaculture. Therefore, the present study was conducted to isolate and characterize *Bacillus subtilis* from rainbow trout intestine and evaluate its probiotic potential. The study was also undertaken to assess the efficacy and potency of the isolated *B. subtilis* on trout growth, immunity and fish resistance to *Aeromonas hydrophila* infection.

Materials and methods

Isolation and characterization of *Bacillus* isolates from rainbow trout intestine

Thirty healthy juvenile rainbow trout (~20–40 g), were randomly obtained from trout farms in Chaharmahal and Bakhtiari, Iran, and transferred to the microbiology laboratory, faculty of natural resources, University of Shahrekord. After fish were euthanized with clove oil, their hindguts were aseptically sampled. For bacilli isolation, 1 g of intestinal content from each fish specimen was removed under aseptic conditions, suspended in 9 ml sterile saline and homogenized for 1 min in

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stomacher. Tenfold serial dilutions of the test samples were conducted, spread on TSA plates, in duplicates, and incubated at 22°C for 48 h. Phenotypic characterization was conducted *viz*., Gram's and spore staining, catalase and carbohydrate (glucose, arabinose, xylose, sucrose, mannose, maltose) fermentation assays (Nair *et al*., 2020; Liu *et al*., 2020). All bacterial isolates were preserved at -80ºC in 50% glycerol stocks and were inoculated into freshly prepared broth when necessary. The isolated bacilli strains were identified at the genus level by performing 16S ribosomal RNA gene analysis using the F (5′-AGAGTTTGATCCTGGCTCAG-3′) and R (5′-TACGGTTACCTTGTTACGACTT-3′) universal primer sets. The obtained data were submitted to BLAST searches of the NCBI GenBank database as described by Altschul *et al*. (1990). Alignments for phylogenetic trees were made using crustal software (Thompson *et al*., 1997). The phylogenetic tree was constructed by a maximum likelihood method using MEGA software (Version 11) (Kaur *et al*., 2018). The sequences of the strains resemble *B. subtilis* was carried out.

In vitro bioassays of isolated *Bacillus* isolates from trout intestine

Haemolytic activity

Hemolytic activity of the selected bacilli strain were assessed by streaking them on sheep blood agar plate (pH 7.2) incubated for 48h at 37°C. The plates were examined for α-hemolysis, β-hemolysis or non-hemolytic properties (Kavitha *et al*., 2018).

Antibiotic susceptibility

Antibiotic susceptibility of the selected bacilli isolates was tested against eight antibiotics consisting of ampicillin (AM 10), gentamicin (GM 10), erythromycin (E 15), tetracycline (TE 30), ciprofloxacin (CP 5), trimethoprim (SXT 1.25/23.75), florfenicol (FF 30) and enrofloxacin (NFX 5) following the disc-diffusion method and the antibiotic sensitivity was interpreted based on the zone of inhibition (mm) according to the Clinical and Laboratory Standard Procedure (CLSI) guidelines (CLSI M31-A3) (Khan *et al*., 2021).

Physicochemical tolerance assays

Tolerance of *Bacillus* species against various physiochemical parameters including temperature, pH, and bile salts was carried. The pH tolerance was

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determined at pHs 1.5, 3, 4.5, 6, 7.5 and 9 by adjusting the pH of broth medium with 1N HCl or 1N NaOH at 37°C for 24 hours. Bile salt resistance of the isolates was determined by overnight cultures of the bacterial strains inoculated in TSB broth containing 2.5, 5.0, 7.5, and 10% of crude bile salts and 2.5 and 5% of artificial bile salts followed by incubation at 37°C for 3 and 6 h. Temperature tolerance was determined by incubating the inoculated broth culture for 24 h at 10, 15, 20 and 30ºC. The growth in all tests was monitored by measuring optical density at 620 nm followed by a viable cell count, and the assays were repeated three times (Kavitha *et al*., 2018).

Detection of biofilm formation (Congo red agar method)

This method is based on the characteristic morphology of biofilm-forming bacteria on Congo red medium. The bacilli isolates were streaked on the Muller Hinton agar (HIMEDIA) supplemented with 0.8 g/L of Congo red dye and incubated for 48 h at 37°C. The production of black colonies with a dry crystalline consistency indicated the biofilm formation and non-biofilm-producing strains develop red colonies (Ramesh *et al*., 2015).

Extracellular enzyme production

Extracellular enzyme-producing capacities of the bacilli strains were qualitatively assessed on selective media plates. Briefly, peptone–gelatine agar plates were inoculated with the isolated strains and incubated at 30°C for 48 h followed by flooding with 15% HgCl2; the clear zone around the colony indicates an affirmative test for proteinase. Starch enriched (1%) agar plates were incubated for 24 h at 30°C and afterword flooded with Lugol's iodine solution; the appearance of whitish-yellow discoloration over plate indicates positive amylase test. Whitish opaque coloration around the bacterial colony over tributyrin (1%) agar gives intimation of lipolytic enzymes produced by the bacteria. Carboxymethyl cellulose agar plates were incubated for 48 h at 30°C and flooded with Gram's iodine solution; the whitish appearance on the periphery of colony indicates cellulose by the bacilli isolates (Emam *et al*., 2020).

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Auto-aggregation

Auto-aggregation of the selected *Bacillus* strains was analyzed according to the odified method by Lee *et al*. (2017). Bacterial cells were harvested by centrifugation at 9400 g for 3 min, washed with PBS twice, re-suspended in the supernatant and then vortexed for 30 s. The absorbance was measured at 600 nm using a spectrophotometer after 2 h. Auto-aggregation (%)=(1-At/A0)×100; A0=Absorbance at 0 h at 600 nm; At=Absorbance at 2 h at 600 nm.

Cell surface hydrophobicity assay

Cell surface hydrophobicity of selected isolates was determined according to Thapa *et al*. (2004). Toluene, chloroform, and ethyl acetate were used to detect the bacilli isolates surface hydrophobicity. The overnight grown cells were collected by centrifugation at 6000g, washed three times with PBS, re-suspended in 10 ml Ringer's solution, and optical density at 600 nm (A0) was measured as control. In tested sample, the cell suspension was mixed with equal volume of solvent, vortexed for 2 min and kept at room temperature for 30 min. The aqueous phase was removed and absorbance was measured at 600 nm (A1). The hydrophobicity of bacterial adhesion to solvent was calculated using the formula: (1−A1/A0) × 100. Percentage values lower than 50% were considered as hydrophilic and values higher than 50% were considered as hydrophobic (indicating the nature of cell surface). The percent hydrophobicity was compared with the positive control. Among the selected isolates, those with comparatively a better survival capacity at digestive transit and hydrophobicity were screened for their adhesion and invasion abilities (Kavitha *et al*., 2018).

Antagonistic activity

Antagonistic effect of bacilli isolates against *Aeromans hydrophila* we examined using agar-well diffusion assay. Cultures of *Bacillus* isolates were first at an appropriate temperature for 24 h. A 20 mL of tryptic soy agar (TSA) was poured into each sterile 100 mm-diameter petri dish. Aliquot of 40 µL of *A. hydrophila* suspension initially adjusted 0.5 McFarland standard was poured on TSA plate, and wells of 6 mm-diameter were punched in the agar with a sterile steel borer. The *Bacillus* cultures were centrifuged at 6000 g for 15 min to remove cell debris, and 10 µL of the supernatant was added into each

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well of agar plate. The inoculated plates were incubated for 24 h at 25℃, before the diameter of the growth zone being measured with the caliper as mm (Balouiri *et al*., 2016).

In vivo bioassays

Safety assay

Twenty healthy rainbow trout previously adapted to new conditions were anesthetized with clove essential oil (50 mg/L) and bacilli isolate identified as *Bacillus subtilis* (isolate no. 6) was intraperitoneally injected at 10^7, 10^8 cfu/fish. The control group was injected with 0.1 mL of sterile PBS/fish. The mortality and disease symptoms were monitored up to 14 days followed by a bacterial culture and Gram staining from the spleen and kidney of tested fish if any.

Diet preparation

Bacillus subtilis isolate no. 6 was grown into TSB at 30°C, 48 h. The cell suspensions were centrifuged at 3000 g for 30 min. The *B. subtilis* spores was prepared in PBS (3×10^9 CFU/ml) after two washes with sterile PBS. The suspension was sprayed on Faradaneh rainbow trout commercial feed (Faradaneh Comp. Shahrekord), and to ensure the number of live bacterial cells in a gram feed, sampling and bacterial counting of the resulting feed was carried out using vial plate count.

Fish and feeding

Three hundred healthy rainbow trout were transferred to department of fishery, faculty of natural resources, university of Shahrekord, and were adapted to new condition for two weeks. Initial weight of individual fish was carried out after 24 h of stopping-feeding and anaesthetizing with clove oil. Two experimental diets each in three replicates (each replicate 50 fish) were formulated: diets containing *B. subtilis* isolate no.6 at 5 x 10^7 cfu/g feed and 1 x 10^8 cfu/g feed, and the experiment was run for 60 days. The control group was fed using basal feed sprayed with PBS. Water quality including temperature, pH, dissolved oxygen, nitrite, unionized ammonia were 12ºC, 8 mg/L, 7.6, <0.1 mg/L and 0.01 mg/L, respectively.

Sampling

At the end of the feeding trial, all fish in each tank were weighed to calculate growth and nutritional indices. Three fish per tank (9 fish per treatment) were randomly obtained, anaesthetized with

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clove essential oil, and blood samples were taken from the caudal vein with heparinized syringes to determine. Additionally, blood samples were taken from the caudal vein of three fish from each tank using non-heparinized syringes, allowed to clot at room temperature for 30 min, and the serum was separated by centrifugation for 10 min at 5000 g and stored at -70°C for analysis of innate immune response parameters (Cha *et al*., 2013).

Growth indices

The growth performance was calculated on day 30 and 60 of feeding using mathematical growth models as followed:

SGR (Specific Growth Rate) (%/day) = $100\times [(LnW_f - LnW_i)/ T]$
FCR (Feed Conversion ratio) = $F / (BW_f - BW_i)$
PER (Protein Efficiency Ratio) = $(BW_f - BW_i)/PI$
WG (Weight Gain) = $(BW_f - BW_i) \times 100$
SR (Survival Rate) = $(N_t/N_0) \times 100$

Where: LnW_f is the natural logarithm of final weight and LnW_i is the natural logarithm of initial weight and 'T' represents the days of culture; BW_f : Final Body Weight, BW_i: Initial Body Weight ; sum T: Temparature (°C) and D: the days of culture; PI: protein intake (gram); N_t: Total fish No. at the end of the trial, N_0: Initial No. (Sahraei *et al*., 2019).

Hematological parameters

Blood samples were diluted with appropriate diluting fluids and RBC and WBC counts were determined using improved Neubauer hemocytometer (Blaxhall and Daisley, 1973). Hemoglobin concentration (Hb) was measured spectrophotometrically (Jenway 6400, UK) at 540 nm by the cyanomethemoglobin method (Rifai, 2017). Hematocrit percentage (Hct %) was measured with the micro-centrifuge method (Micro-hematocrit centrifuge, 346, UNIPAA, Poland) for 10 min in duplicate.

Biochemical parameters

Serum biochemical parameters were determined spectrophotometrically using commercial diagnostic kits (Pars Azmoon Co., Tehran, Iran) and the following equation: (Absorption of sample / Absorption of standard) × concentration of the standard (Ghanei-Motlagh *et al*., 2021).

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Intestinal enzyme activity

Fish intestinal tract was homogenized according to the methods described by Gisbert *et al*. (2016), and samples were kept in −80°C for further analysis. All analyses were performed using standard methods for the selected digestive enzymes including trypsin and chymotrypsin (Erlanger *et al*., 1961) and bile salt-activated lipase (Iijima *et al*., 1998). Lipase activity was determined by measuring fatty acids released following enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil (Didinen *et al*., 2014) and amylase activity was quantified using starch as a substrate at 540 nm (Eldar *et al*., 1999). Protease activity was measured as described previously (Raissy *et al*., 2018) using casein hydrolysis at pH: 8.0. Protease activity was measured using casein (Sigma–Aldrich) as the substrate and then the product reaction with Folin's reagent (Anson, 1938; Sun *et al*., 2012). Digestive enzyme activities (i.e., alkalin phosphatase, lipase, amylase, trypsin and protease) were presented as specific activity (U mg^{-1} protein min^{-1}) that shows the amount of product formed by an enzyme at a given time.

Lysozyme activity

Serum lysozyme activity was determined by the turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich). According to the method of Sharifuzzaman and Austin (2009), 0.02 M sodium phosphate buffer (SPB) (pH = 5.8, Sigma–Aldrich) was used. The SPB-free serum sample was applied as a negative control. Results were obtained at 450 nm and expressed in the unit of lysozyme per ml serum when causing a reduction of 0.001 per min at 22°C (Mohammadian *et al*., 2019).

Serum complement activity

Serum alternative pathway complement activity (ACH50) was assayed following the method of Yano (1992), using rabbit red blood cells (RaRBC). The test serum was diluted appropriately with EGTA–Mg–GVB and RaRBC. The tubes were held at 0 °C to retard the action of complement until all tubes in a set of five serum samples were prepared. Subsequently, 100 mL of RaRBC suspension was added to each tube and incubated at 20°C for 90 min with occasional shaking. At this point, 5 mL of saline was added to each tube and centrifuged at 1600 g for 5 min. The absorbance of the supernatant was read

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at 414 nm. The absorbance of the supernatant was read at 414 nm. The degree of hemolysis was calculated by dividing the corrected A414 value by the corrected A414 of the 100% hemolysis control (Amar *et al*., 2000).

Antioxidant assays

The serum catalase (CAT) activity was measured by determining the reduction in absorbance at 240 nm in the presence of 13.2 mM H2O2 in 50 mM phosphate buffer (pH 7.0) and 100 µL of serum (Aebi, 1984). A control containing 50 mM phosphate buffer (pH 7.0) and 100 µL of serum was used to determine the CAT activity. The activity of serum superoxide dismutase (SOD) was measured by using 1.2 mL sodium pyrophosphate buffer + 100 µL phenazine methosulfate + 300 µL nitroblue tetrazolium (McCord and Fridovich, 1969). Two hundred µL of the serum was added to the assay mixture, and the total volume increased to 2.8 mL with distilled water. Then 200 µL of NADH was added to the previous mixture to initiate the reaction at 30◦C for 90 s. Then the reaction was halted by adding 1.0 mL of glacial acetic acid. Then 4.0 mL of n-butanol was added to the mixture, shaken and stood for 10 min, and centrifuged (5000 *g*, 10 min). The inhibition activity of SOD spectrophotometrically was measured at 560 nm in the butanol layer for 60 s. The GSH level was measured through the dithionitrobenzoic acid recycling method described by Ellman (1959).

RNA extraction and cDNA synthesis

Total RNA was extracted from 6 intestine samples for each treatment on day 30 and 60, using the Sam Bio RNA extraction kit according to the manufacturer's procedure (Qiagen). The concentration of extracted RNA was calculated at a wavelength of 260 nm using nano-drop spectrophotometry (Eppendorf, Germany). To detect the purity of RNA, the optical density (OD) was determined at 260/280 nm and samples having a ratio more than 1.8 were used for the cDNA synthesis.

Real-time quantitative PCR

To evaluate the expression levels of tumor growth factor (TGF), interleukin-1-β (IL-1β) and heat shock protein 70 (HSP70) in the intestine and kidney, real-time PCR was performed using qPCRBIO SyGreen 1 step detect LO-ROX (PCR BIOsystems simplifying research) on a Light cycler Detection

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System (Roche, USA). Relative expression levels of the all transcripts were compared to EFI as a housekeeping gene. Specific sets of primers (Pioneer, South Korea) were designed based on rainbow trout (Table 1).

Table 1. Sequences of oligonucleotide primers used in this study.

Sequence name	Sequence
EF1-F	CAAGGATATCCGTCGTGGCA
EF1-R	ACAGCGAAACGACCAAGAGG
Hsp70 F	TCAAGAGGAAACACAAGAAGGA
Hsp70 R	TGGTGATGGAGGTGTAGAAGTC
TGF F	TCCGCTTCAAAATATCAGGG
TGF R	TGATGGCATTTTCATGGCTA
IL-1β F	CTCTACCTGTCCTGCTCCAAA
IL-1β R	ATGTCCGTGCTGATGAACC

Reactions were performed in a 12.5 all mixture containing 6.25 all qPCRTM Green Master Mix (2X), 0.25 µL of each primer (10 µM), 3 µL (100 ng) cDNA, and 2.75 µL nuclease-free water. The PCR protocol consisted of a 5 min denaturation at 94℃ followed by 45 cycles at 94℃ for 15 sec and 60℃ for 30 sec. Reactions were performed in triplicate. Two separate reactions without cDNA or with RNA were performed as control groups in parallel with experimental groups. According to the comparative 2-ΔΔCt method, the relative quantification was performed using light cycler 96R software. Validation of assay to check that the primers for the EFI had similar amplification efficiencies was carried out as described previously (Mohammadian *et al*., 2018). All qPCR analysis was performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guideline (Bustin *et al*., 2009).

Resistance to *Aeromonas hydrophila* infection

At the end of the experiment, thirty fish from each treatment were first starved for 24 h before being anesthetized with clove essential oil and were injected intraperitoneally with 0.1 mL of *A. hydrophila* (LD_{50} equal to 1×10^6 CFU/ml). The challenged fish were

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observed for 14 d for recording of the clinical signs, post-mortem lesions, and daily mortality. The Relative percentage of survival (RPS) was determined using the following formula:

RPS = [1 - [Percent mortality in treated group/Percent mortality in control group] × 100

Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA). Significant differences among the means were processed by means of Tukey multiple range test. The level of significant difference was set at $p<0.05$ and all statistical analyses were carried out using the SPSS 27.0 software (SPSS, Chicago, IL, USA).

Results

Isolation and characterization of bacilli bacteria from trout gastrointestinal tract

Six bacilli isolates were obtained from rainbow trout, and they were gram positive, catalase positive and spore producing *Bacillus*. These bacilli isolates were positive for production from glucose, arabinose, mannose, sucrose, xylose and maltose (Table 2). The electrophoresis of PCR product exhibited bands with 1554 bp that is identical to *Bacillus* genus (Fig. 1). The BLAST analysis of the 16S rRNA sequences from the isolates 5 and 6 revealed high homology (98%–100%) with *B. subtilis* (Table 1). The sequences of isolate number 2 resulted in identification of *Bacillus simplex*, while the sequences of isolates numbers 1, 3 and 4 were not successful.

Table 2. Phenotypic characteristics of the isolated bacilli from trout intestine. + = positive result; – = negative result.

Assay	1	2	3	4	5	6
Catalase	+	+	+	+	+	+
Malachite green	+	+	+	+	+	+
Glucose oxidation	+	-	-	+	+	+
Arabinose oxidation	+	-	+	+	-	+
Mannose oxidation	+	+	+	+	+	-
Sucrose oxidation	+	+	+	+	-	+
Xylose oxidation	+	+	+	-	-	+
Maltose oxidation	+	+	+	+	+	-

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Phylogenetic analysis

Comparing the isolated Bacillus subtilis strain 6 with the reported strains from different regions exhibited most similarity with the strains of *Bacillus subtilis* reported from India, Japan and South Korea (Fig 2).

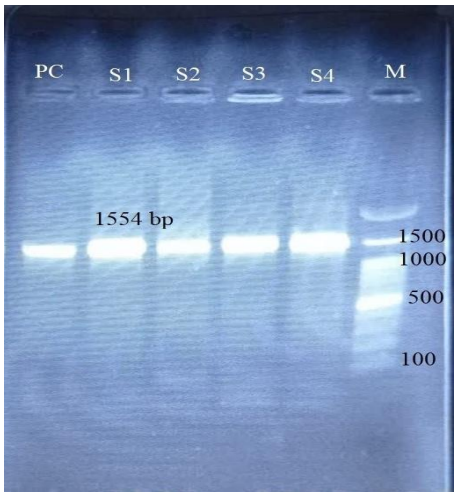

Figure 1. Electrophoresis of PCR product for isolates 1, 2, 5, and 6 of *Bacillus* strains. Line PC=positive control, Lines 1-4= samples isolates (test samples), M= marker.

Hemolytic activity

The bacilli strains numbers 1, 2, 3, 5 and 6, were non-hemolytic (γ-hemolysis), while strain 4 was hemolytic (β-hemolysis).

Antibiotic susceptibility

Isolates 1, 4, 5 and 6 showed sensitivity to all tested antibiotics (Table 3). Isolate 2 was sensitive to tetracycline, trimethoprim and florfenicol, and isolate 3 exhibited a sensitivity to florfenicol.

Bile tolerance

All *Bacillus* isolated demonstrated a positive growth at all concentrations of crude bile salt, but bacilli isolate numbers 1 and 6 exhibited a higher growth at 2.5, 5 and 10% bile salt than other isolates (p<0.05). Also, isolate number 6 demonstrated a higher growth at 7.5% salt bile than other isolates (Table 4). In addition, all bacilli isolates were able to survive in commercial bile salt, but isolate 6 demonstrated a higher survival at 2.5% and 5% (2.56±0.20 and 2.03±0.15) than other isolates (Table 5).

Table 3. Antibiotic susceptibility of *Bacillus* strains obtained from trout intestine.

Antibiotic	Antagonistic (mm)					
	1	**2**	**3**	**4**	**5**	**6**
Ampicillin (AM 10)	4	1.5	3.5	4	3	4
Gentamicin (GM 10)	4	3	3	4	4	4
Erythromycin (E 15)	4	3.3	3	4	4	4
Tetracycline (TE 30)	4	4	3	4	4	4
Ciprofloxacin (CP 5)	4	3	3	4	4	4

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Table 3 continued:						
Trimethoprim (SXT 1.25/23.75)	4	4	3.5	4	4	4
Florfenicol (FF 30)	4	5	4	4	4	4
Enrofloxacin (NFX 5)	4	3	3.5	4	4	4

Table 4. Bile salt (Crude) tolerance analysis of Bacillus isolates in terms of CFU ml^{-1} ($\times 10^8$). Each value is the mean ± standard deviation of three separate experiments.

Bacillus No.	**Crude bile Salt (%)**				
	0% (PBS)	**2.5%**	**5%**	**7.5%**	**10%**
1	2.96±0.15 ^{a}	2.83±0.15 ^{a}	2.66±0.15 ^{a}	1.86±0.15 ^{b}	1.76±0.15 ^{a}
2	0.36±0.05 ^{c}	0.26±0.05 ^{d}	0.16±0.05 ^{c}	0.11±0.02 ^{c}	0.00±0.00 ^{b}
3	0.33±0.05 ^{c}	0.23±0.05 ^{d}	0.11±0.01 ^{c}	0.10±0.00 ^{c}	0.10±0.01 ^{b}
4	1.83±0.20 ^{b}	1.83±0.15 ^{b}	1.73±0.15 ^{b}	1.73±0.15 ^{b}	1.73±0.20 ^{a}
5	2.66±0.15 ^{a}	0.90±0.10 ^{c}	0.05±0.01 ^{c}	0.02±0.00 ^{c}	0.01±0.00 ^{b}
6	2.76±0.15 ^{a}	2.96±0.15 ^{a}	2.60±0.10 ^{a}	2.30±0.20 ^{a}	1.66±0.15 ^{a}

Table 5. Bile salt tolerance analysis of *Bacillus* isolates in terms of CFU ml^{-1} ($\times 10^8$). Each value is the mean ± standard deviation of three separate experiments.

Bacillus No.	**Bile salt (%)**		
	0% (PBS)	**2.5**	**5**
1	1.76±0.10 ^{d}	1.30±0.11 ^{c}	1.16±0.15 ^{cde}
2	1.56±0.15 ^{de}	1.46±0.15 ^{b}	1.40±0.10 ^{efg}
3	1.70±0.10 ^{cde}	1.53±0.15 ^{c}	1.26±0.15 ^{def}
4	2.96±0.15 ^{b}	1.40±0.10 ^{bc}	1.16±0.05 ^{fg}
5	1.40±0.10 ^{de}	1.70±0.15 ^{bc}	1.33±0.20 ^{cde}
6	3.16±0.15 ^{b}	3.00±0.10 ^{a}	2.56±0.20 ^{a}

pH tolerance

All isolates could not survive in pH 1.5 but isolate 6 survived at pHs 3, 4.5 and 6 better than others. The optimum condition to grow for most isolates was pH 6, but isolate 4 prefer alkaline condition. All *Bacillus* strains showed a significant difference in different pH values ($P<0.001$) (Table 6).

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Table 6. pH tolerance analysis of *Bacillus* isolates in terms of CFU ml^{-1} ($\times 10^{7}$). Each value is the mean ± standard deviation of three separate experiments.

Bacillus No.	pH value					
	1.5	3	4.5	6	7.5	9
1	0	0.03±0.00 ^{e}	0.03±0.00 ^{f}	0.11±0.00 ^{f}	0.03±0.00 ^{h}	0.02±0.00 ^{h}
2	0	0.01±0.00 ^{f}	0.02±0.00 ^{f}	0.03±0.00 ^{i}	0.03±0.00 ^{h}	0.01±0.00 ^{h}
3	0	0.00±0.00 ^{f}	0.00±0.00 ^{a}	0.01±0.00 ^{i}	0.01±0.00 ^{i}	0.01±0.00 ^{h}
4	0	0.28±0.00 ^{c}	0.25±0.00 ^{c}	0.36±0.00 ^{c}	0.52±0.00 ^{b}	0.51±0.00 ^{b}
5	0	0.05±0.00 ^{e}	0.04±0.00 ^{e}	0.05±0.00 ^{h}	0.09±0.00 ^{g}	0.01±0.002 ^{h}
6	0	0.51±0.01 ^{b}	0.56±0.00 ^{a}	0.62±0.00 ^{a}	0.82±0.00 ^{c}	0.46±0.00 ^{c}

Temperature tolerance

Isolate number 6 gave a higher growth at 10°C than other isolates (p<0.05), but isolate number 4 presented a better growth at 20°C than other isolates (p<0.05). All isolate number 2 provided a higher growth at 15°C or 30°C than other isolates (Table 7).

Table 7. Temperature tolerance analysis of *Bacillus* isolates (10^{6} CFU mL^{-1}). Each value is the mean ± standard deviation of three separate experiments.

Bacillus No.	Temperature (°C)			
	10	15	20	30
1	0.02±0.00 ^{g}	0.01±0.00 ^{h}	0.91±0.00 ^{b}	0.17±0.01 ^{f}
2	0.66±0.01 ^{c}	0.96±0.01 ^{a}	080±0.00 ^{e}	1.41±0.01 ^{a}
3	0.01±0.00 ^{g}	0.01±0.00 ^{h}	0.03±0.00 ^{ef}	0.05±0.00 ^{g}
4	0.16±0.01 ^{e}	0.73±0.01 ^{c}	1.04±0.00 ^{a}	1.00±0.02 ^{b}
5	0.43±0.01 ^{d}	0.33±0.01 ^{f}	0.20±0.01 ^{d}	0.23±0.01 ^{e}
6	0.76±0.02 ^{b}	0.82±0.02 ^{b}	1.04±0.20 ^{a}	0.53±0.01 ^{d}

Detection of biofilm formation (Congo red agar method)

Congo red agar method was used to screen and ascertain the ability of the isolates to produce biofilm. Only isolate 2 formed black colonies indicating positive biofilm production but others were negative for biofilm production.

Extracellular enzyme production

Extracellular enzyme activities of bacilli isolates are shown in Table 8. Isolate number 6 was positive for all tested enzymes (Fig. 3).

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Table 8. Extracellular enzyme activities of *Bacillus* strains numbers 1-6 obtained from trout intestine.

Enzyme	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Starch (amylase)	-	+	+	+	+	+
Peptone (gelatinase)	-	-	+	+	-	+
CMC (cellulose)	-	-	-	+	-	+
Lipase	-	-	+	+	-	+
Skim milk (protease)	+	-	+	+	-	+

Symbol '+' denotes a positive result, '–' denotes a negative result.

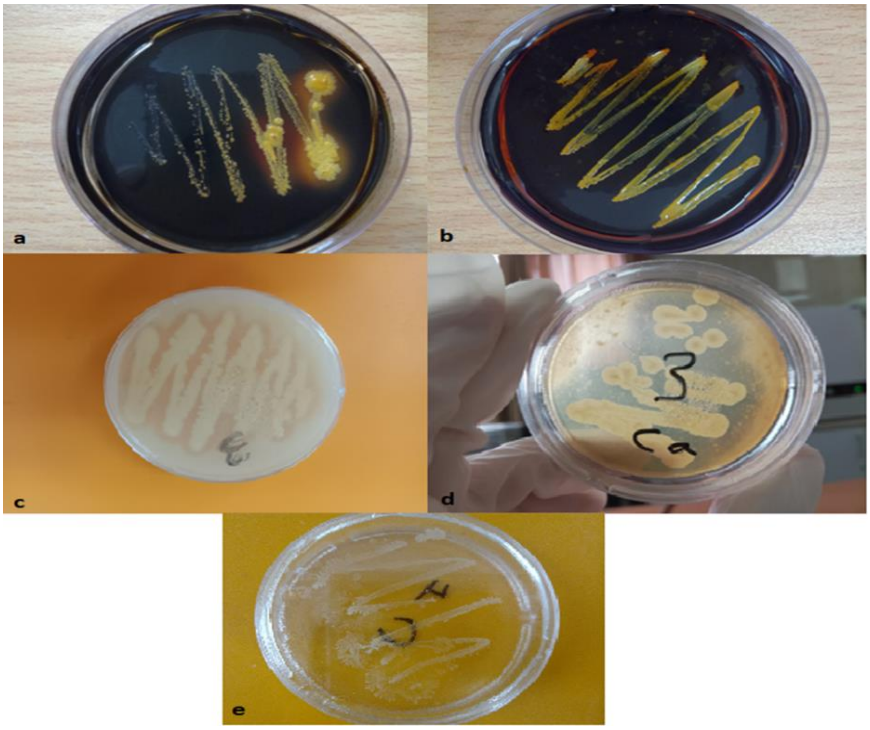

Figure 3. Qualitative examination of enzymes production of bacterial isolates; a: bacteria with amylase enzyme (fluorescent yellow halo around the bacterial growth line), b: bacteria without amylase enzyme (the growth line only turned yellow after pouring Lugol, but has no fluorescent state), c: lipase positive bacteria, d: caseinase positive bacteria, e: cellulase positive bacteria.

Auto-aggregation

Auto-aggregation assay which is strongly correlated with cell adhesion to the digestive tract revealed that isolate number 6 demonstrated an auto-aggregation (94.8%) higher than other isolates followed by isolates 5 and 2, respectively (Table 9).

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Table 9. Mean auto-aggregation of *Bacillus* isolates obtained from trout intestine.

Bacillus No	Auto-aggregation (%)
1	33.70±0.36 [e]
2	56.53±0.50 [c]
3	33.66±0.35 [e]
4	46.56±0.40 [d]
5	85.46±0.50 [b]
6	94.60±0.52 [a]

Cell hydrophobicity

Cell hydrophobicity of the isolates to ethyl acetate, chloroform and xylene was tested to determine the adhesion capability of the bacterial to cell surfaces, and the results exhibited that cell hydrophobicity of the isolates 5 and 6 was significantly higher than other isolates (p<0.05) (Table 10).

Table 10. Cell hydrophobicity of bacilli isolates obtained from trout intestine. Mean ± SD (n = 3).

Bacillus subtilis no.	Solvent		
	Toluene	Choloroform	Ethyl acetate
1	26.67±1.52 [d]	15.33±1.52 [d]	22.33±2.51 [f]
2	42.33±2.51 [c]	32.67±2.08 [c]	25.33±2.51 [e]
3	35.00±3.00 [c]	27.00±2.00 [c]	32.33±2.51 [d]
4	52.00±2.00 [b]	42.33±2.51 [b]	54.00±2.00 [c]
5	81.33±3.78 [a]	73.00±2.64 [a]	64.00±2.64 [b]
6	83.33±3.21 [a]	68.67±2.51 [a]	74.67±2.51 [a]

Antagonistic activity

Antagonistic assay toward *A. hydrophila* confirmed that Bacillus isolate No. 6 revealed the highest resistance to *A. hydrophila* in vitro conditions (Fig. 4).

Safety status

All of rainbow trout no mortality was seen in the fish injected with bacilli isolate no 6 14 days post-injection

Growth performance

The values of weight gain, specific growth rate and protein efficiency in fish fed *B. subtilis* at 1 x 10^8 cfu/ g feed were higher than fish fed 5x10^7 cfu/g feed and control fish, while food conversion ratio was lower in fish fed 1 x 10^8 cfu/g feed than other treatment and control (p<0.05) (Table 11).

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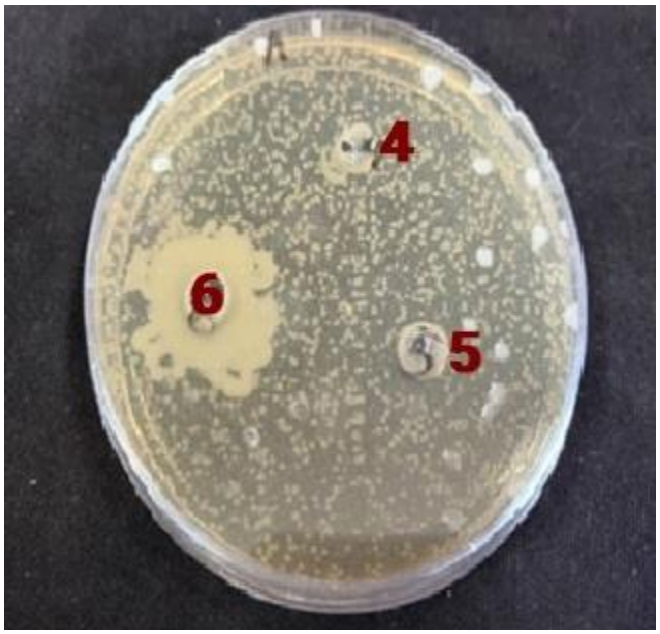

Figure 4. Antagonistic activity of *Bacillus* isolates 4-6 against *A. hydrophila*.

Table 11. Growth performance of rainbow trout fed *Bacillus subtilis* at 5 x10^7 and 1 x 10^8 cfu/g feed for two months at 2C. Values (mean ± SD, n=3) with different superscripts in each row are significantly different (P < 0.05). IW = Initial weigh, WG (%) = Weight gain; PER = Protein Efficiency Ratio; SGR = Specific growth rate; FCR = Feed Conversion ratio; SR = Survival rate.

Time	Growth factor	*Bacillus subtilis* (5 x 10^7CFU/g feed)	*Bacillus subtilis* (1 x 10^8 CFU/g feed)	Control
Day 30	IW	23.20±0.20 ^{a}	22.43±0.35 ^{ab}	21.76±0.25 ^{b}
	WG (%)	66.00±1.00 ^{b}	108.00±1.00 ^{a}	67.67±1.52 ^{b}
	PER	2.66±0.11 ^{c}	4.06±0.01 ^{a}	2.73±0.01 ^{b}
	SGR	1.08±0.01 ^{c}	1.81±0.01 ^{a}	1.41±0.01 ^{b}
	FCR	0.83±0.01 ^{a}	0.67±0.02 ^{b}	0.87±0.02 ^{a}
	SR (%)	90	100	90
Day 60	WG (%)	83.67±1.15 ^{b}	87.67±0.57 ^{a}	50.67±0.57 ^{c}
	PER	4.06±0.01 ^{c}	5.03±0.11 ^{a}	2.40±0.10 ^{b}
	SGR	2.36±0.05 ^{c}	2.83±0.05 ^{a}	1.73±0.05 ^{b}
	FCR	0.64±0.01 ^{b}	0.53±0.02 ^{c}	1.11±0.03 ^{a}
	SR (%)	100	100	100

Hematological parameters

Inclusion of *B. subtilis* at 5 x 10^7 and 1 x 10^8 cfu/g in trout feed demonstrated an increase in populations of white blood cells (WBC) and hemoglobin value compared to control fish (P<0.05). These

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values showed a variation on day 60 of feeding but still were higher than control fish (Table 12). The value of red blood cells (RBC) on day 30 of feeding was significantly higher than control fish, but its level on day 60 of feeding exhibited higher than fish fed *B. subtilis* at $5x10^7$ cfu/g feed.

Table 12. Hematological parameters of rainbow trout serum fed with diets containing indigenous *B. subtilis* for two months at 12ºC.

Time	Treatment	Hemoglobin	WBC ($\times10^4$/mm^3)	RBC ($\times10^6$/mm^3)
Day 30	*B. subtilis* 5 x 10^5 cfu/g feed	9.66±0.15 [a]	8.23±0.10 [a]	1.29±0.01 [b]
	B. subtilis 1 x 10^8 cfu/g feed	9.23±0.15 [b]	7.84±0.04 [b]	1.59±0.01 [a]
	Control	8.33±0.15 [c]	7.11±0.03 [c]	1.11±0.01 [c]
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	8.26±0.15 [a]	8.16±0.06 [a]	1.13±0.01 [c]
	B. subtilis 1 x 10^8 cfu/g feed	7.66±0.15 [b]	8.08±0.03 [a]	1.54±0.01 [a]
	Control	7.66±0.57 [b]	7.35±0.03 [b]	1.33±0.02 [b]

Biochemical parameters

There was no significant difference in AST value between two treatments, but both were significantly lower than control fish (p<0.05). ALT value in fish fed 5 x 10^7 cfu/g feed was higher than fish fed $1x10^8$ cfu/g feed and control till day 30 of feeding (p<0.05) but it raised to higher level in control fish than the treatments. The level of cholesterol and triglyceride in both treatments was lower than control fish (P<0.0). No difference was seen in glucose level among treatments (Table 13).

Table 13. Blood biochemical parameters (U mg protein^{-1}) of rainbow trout fed with diets containing indigenous *B. subtilis* for two months at 12ºC.

Time	Treatment	AST	ALT	Cholesterol	Triglyceride	Glucose
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	27.66±1.51 [a]	50.66±2.51 [a]	210.33±0.57 [b]	182.33±6.02 [a]	7.67±0.57 [a]
	B. subtilis 1x 10^8 cfu/g feed	27.33±1.16 [a]	40.33±1.52 [b]	201.00±1.00 [c]	122.00±4.58 [c]	6.67±0.57 [a]
	Control	31.33±6.11 [a]	31.00±1.73 [c]	363.00±1.00 [a]	157.66±6.11 [b]	6.67±0.57 [a]
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	26.33±1.27 [a]	19.66±1.52 [b]	205.33±1.52 [b]	107.66±7.09 [b]	14.00±1.00 [a]
	B. subtilis 1x 10^8 cfu/g feed	23.00±1.60 [a]	14.66±1.52 [c]	202.00±1.00 [b]	78.00±5.56 [c]	13.00±1.00 [a]
	Control	39.33±2.50 [b]	32.66±1.52 [a]	231.66±1.52 [a]	163.00±4.58 [a]	15.00±1.00 [a]

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The levels of albumin and total protein at the end of the trial in fish fed 1 x10^8 cfu/g feed was higher than fish received 5 x10^7 cfu/g feed and control fish, while globulin value in fish fed 5x10^7 cfu/g feed was higher than fish fed 1 x10^8 cfu/g feed and control fish (p<0.05) (Table 14).

Table 14. Serum proteins levels in rainbow trout fed indigenous *Bacillus subtilis*. Data are presented as the mean ± SE of three samples for 30 and 60 days.

Time	Treatment	Albumin	Globulin	Total Protein
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	2.20±0.14 [a]	1.44±0.48 [c]	3.64±0.40 [b]
	B. subtilis 1 x 10^8 cfu/g feed	1.84±0.58 [b]	2.05±0.68 [a]	3.89±0.77 [a]
	Control	1.60±0.44 [c]	1.56±0.55 [b]	3.16±0.66 [c]
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	1.63±0.57 [c]	2.13±0.54 [a]	3.76±0.28 [b]
	B. subtilis 1 x 10^8cfu/g feed	2.54±0.62 [a]	1.97±0.66 [b]	3.82±0.30 [a]
	Control	1.85±0.33 [b]	1.69±0.77 [c]	3.54±0.74 [c]

Innate immune parameters

The values of complement in both treatments were higher than control fish (p<0.05), but there was no difference between two treatments (p>0.05). The lysozyme value in fish fed 1x10^8 cfu/g feed was higher fish fed 5x10^7 cfu/g feed and control fish (p<0.05) (Table 15).

Table 15. Lysozyme and complement activities in rainbow trout serum fed indigenous *Bacillus subtilis*. Data are presented as the mean ± SE of three samples for 30 and 60 days.

Time	Treatment	Complement activity	Lysozyme
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	136.36±0.45 [a]	318.20±43.65 [b]
	B. subtilis 1 x10^8 cfu/g feed	135.70±0.36 [a]	336.70±38.23 [a]
	Control	131.73±0.35 [b]	303.40±30.33 [c]
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	129.53±0.35 [a]	418.10±25.95 [b]
	B. subtilis 1 x 10^8 cfu/g feed	128.76±0.35 [a]	458.90±36.47 [a]
	Control	121.80±0.30 [b]	355.20±72.95 [c]

Antioxidants

The Super oxide dismutase (SOD) activity in both treatments was higher than control fish (p<0.05), but there was no significant difference between two treatments. The activity of catalase and glutathione in fish received *B. subtilis* at 1 x10^8 cfu/g feed was significantly higher than other treatment and control fish (p<0.05) (Table 16).

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Table 16. Antioxidant activity of sera samples of rainbow trout fed different indigenous *Bacillus subtilis*. Data are presented as the mean ± SE of three samples for 30 and 60 days.

Time	Treatment	Super oxide dismutase U mg protein^{-1}	Glutathione (mmol mL^{-1})	Catalase U mg protein^{-1}
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	27.77±2.32 ^{a}	1.46±0.29 ^{b}	0.17±0.07 ^{c}
	B. subtilis 1 x 10^8 cfu/g feed	28.24±4.45 ^{a}	2.72±1.64 ^{a}	0.47±0.21 ^{a}
	Control	16.52±2.06 ^{b}	0.66±0.17 ^{c}	0.33±0.06 ^{b}
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	83.10±17.01 ^{a}	1.48±0.95 ^{c}	0.45±0.25 ^{b}
	B. subtilis 1 x 10^8 cfu/g feed	83.65±7.10 ^{a}	6.93±1.52 ^{a}	0.50±0.19 ^{a}
	Control	19.46±1.68 ^{b}	3.70±1.73 ^{b}	0.11±0.06 ^{c}

Digestive enzymes

Chymotrypsin level in both treatments was lower than control group (p>0.05), but levels of trypsin and lipase in fish received 5 x10^7 cfu/g in feed was higher than fish fed *B. subtilis* at 1x10^8 cfu/g feed and control fish at the end of experiment (p<0.05), while protease and amylase levels in fish fed 1 x10^8 cfu/g feed was higher than other treatment and control (p<0.05) (Table 17).

Table 17. Digestive enzymes of rainbow trout serum fed different indigenous *Bacillus subtilis*. Data are presented as the mean ± SE of three samples for 30 and 60 days.

Time	Treatment	Chymotrypsin (mU protein^{-1})	Trypsin (U mg protein^{-1})	Lipase (U mg protein^{-1})	Protease (U mg protein^{-1})	Amylase (U mg protein^{-1})
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	0.11±0.00 ^{b}	2.19±0.87 ^{a}	2.06±0.03 ^{b}	0.25±0.07 ^{b}	33.71±4.91 ^{a}
	B. subtilis 1 x10^8 cfu/g feed	0.12±0.07 ^{b}	1.72±1.18 ^{b}	2.32±0.30 ^{a}	0.39±0.05 ^{a}	33.68±6.25 ^{b}
	Control	0.20±0.15 ^{a}	1.59±0.17 ^{c}	2.05±0.02 ^{b}	0.13±0.63 ^{c}	24.92±7.72 ^{c}
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	0.18±0.05 ^{c}	2.53±10 ^{a}	2.09±0.05 ^{a}	0.39±0.08 ^{b}	38.01±3.76 ^{b}
	B. subtilis 1 x10^8 cfu/g feed	0.29±0.07 ^{b}	2.50±0.44 ^{b}	2.05±0.03 ^{b}	0.57±0.20 ^{a}	42.73±5.31 ^{a}
	Control	0.38±0.26 ^{a}	1.47±0.17 ^{c}	1.08±0.07 ^{c}	0.17±0.51 ^{c}	16.61±1.47 ^{c}

Relative mRNA expression of immune-related genes

The expression of IL-1β, TGF-β and HSP70 genes in both treatments was higher than control fish (p<0.05), and the

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upregulation of IL-1β and HSP70 in fish fed 1x0^8 cfu/g feed was higher than fish received 5 x10^7 cfu/g feed (p<0.05) (Table 18).

Table. 18. Effect of the experimental treatments on the quantitative expressions of IL-1β, TGF-β and HSP70 extracted from the intestine of rainbow trout (mean±SE) after 30 and 60 days of feeding. Groups with different superscript are significantly different (p<0.05).

Time	Treatment	IL-1β	TGF-β	HSP70
Day 30	*B. subtilis* 5 x 10^7 cfu/g feed	4.47±0.03 ^{b}	8.44±0.10 ^{a}	5.55±0.23 ^{a}
	B. subtilis 1 x10^8 cfu/g feed	8.42±0.28 ^{a}	5.41±0.14 ^{b}	3.98±0.13 ^{b}
	Control	0.99±0.01 ^{c}	1.05±0.07 ^{c}	1.04±0.05 ^{c}
Day 60	*B. subtilis* 5 x 10^7 cfu/g feed	4.05±0.01 ^{b}	5.28±0.04 ^{a}	4.10±0.02 ^{b}
	B. subtilis 1 x10^8 cfu/g feed	7.09±0.01 ^{a}	5.07±0.04 ^{a}	5.11±0.02 ^{a}
	Control	0.99±0.00 ^{c}	1.04±0.06 ^{b}	0.99±0.00 ^{c}

Disease resistance

After being challenged with *A. hydrophila* for 21 days, the cumulative mortality rates of rainbow trout fed the *B. subtilis* diet were all significantly lower (10%) than control fish (40%) (p<0.05), and the *A. hydrophila* was re-isolated as single colonies from the spleens and livers of the moribund or dead fish. The RPS of 75% was obtained for both dosages of *Bacillus subtilis*.

Discussion

Bacillus bacteria are dominated primary inhabitants of both freshwater and marine environments and generally exist more frequently in sediments than in the water column, and thus, they can naturally be ingested by aquatic animals. In this study 6 bacillus strains were isolated from rainbow trout among them isolates 5 and 6 revealed high homologies with *B. subtilis*, and isolate number 2 was identified as *Bacillus simplex*, while the sequences of isolates numbers 1, 3 and 4 were not successful. Based on in vitro bioassays including hemolytic activity, antibiotic susceptibility, bile tolerance, pH tolerance, temperature tolerance, detection of biofilm formation (Congo red test), *and B. subtilis* isolate 6 exhibited a better result as a potential probiotic candidate. Further bioassays including secretion of extracellular enzymes (amylase, lipase, and caseinase cellulose), auto-aggregation, cell hydrophobicity, and antagonistic activity

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toward *A. hydrophila* this B. subtilis (isolate 6) was superior to other bacilli isolates. Therefore this *B. subtilis* strain was selected for further probiotic evaluation by the *in vivo* bioassays.

When the indigenous B. subtilis was examined for the safety status, its injection to rainbow trout at different dosages did not affect the fish and no bacteria was re-isolated from the fish after 2 weeks post- injection. Therefore, this safe B. subtilis isolate was considered for its oral utilization for two months at two dosages, and the results were promising as growth factors including weight gain, specific growth rate in fish fed *B. subtilis* at higher concentration i.e., $1x10^8$ cfu/g feed were significantly higher than fish fed lower concentration i.e., $5x10^7$ cfu/g feed and control fish. Also, nutritional factors such as feed protein efficiency and feed conversion ratio exhibited higher and lower in fish fed $1x10^8$ cfu/g feed than other treatment and control, respectively. Such an improvement in the growth performance of treated trout can in part be due to an increase in activity of digestive enzymes including protease, lipase, amylase, and trypsin measured here in treated trout fed indigenous B. subtilis.

No difference was seen in AST and glucose levels between treatments and control, but ALT showed an enhancement in fish fed a lower dosage of the probiotic. Also, an improvement in albumin and total protein, globulin, and a reduction in cholesterol and triglyceride levels in fish fed the indigenous *B. subtilis*, suggesting no side effect by including this bacilli probiotic in trout fish for two months. Assessment of hematological indices showed that oral administration of indigenous *B. subtilis* at both lower and higher dosages ($5x10^7$ and $1x10^8$ cfu/g feed) enhanced leukocyte populations compared to control fish indicating of positive innate immune-stimulatory effect by this strain of Indigenous *B. subtilis* in trout. Similar findings were seen in hemoglobin value, value of red blood cells was also increased up to day 30 of feeding in both bacilli dosages compared to control fish, but its level feeding exhibited a higher value in fish fed *B. subtilis* at $5x10^7$ cfu/g feed than others. The values of complement in both treatments were higher than control fish with no difference between two treatments, while lysozyme value in fish fed $1x10^8$ cfu/g feed was higher fish fed $5x10^7$ cfu/g feed and control fish. This is

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supported by an enhancement seen in the leukocyte population of treated trout with indigenous *B. subtilis* compared with control fish.

In addition, a higher increase in antioxidant enzymes including SOD, catalase and glutathione particularly at higher bacilli dosage suggesting a well immune-stimulatory effect by inclusion this indigenous in trout diet. Further, both groups of trout fed indigenous B. subtilis particularly at higher dosage exhibited upregulation in expression of IL-1β, TGF-β and HSP70 genes better than control fish, suggesting improvement of trout immune responses after being fed with this indigenous B. subtilis for two months. This finding was supported by significantly higher survival of treated trout (90%) than control fish (60%) after challenge with *A. hydrophila* infection, and RPS of 75% was obtained in both trout treatments within 21 days of challenge.

Several studies have shown the immunostimulatory effects by *B. subtilis* in fish. For instance, in a study by Newaj-Fyzul *et al*. (2007) *B. subtilis* isolated from rainbow trout intestine orally administered in trout for two weeks exhibited a significant enhancement in lysozyme, respiratory burst, phagocytosis, peroxidase activity, and anti-protease activity. The treated fish also showed a higher survival against *Aeromonas* sp. infection than control fish. In addition, rainbow trout fed with *B. subtilis* exhibited higher immunostimulatory effects of granulocyte population, antibody level, and activities of respiratory burst, phagocytosis, as well as a higher survival to *Yersinia ruckeri* infection (Yılmaz *et al*., 2019). Further, erythrocyte level was enhanced in Kutum *(Rutilus frisii kutum***)** orally subjected to *B. subtilis* probiotic (Azarin *et al*., 2015). Furthermore, when species of common carp (*Cyprinus carpio*), grass carp, koi carp, and rohu (*Labeo rohita*) were fed with *B. subtilis* exhibited enhancement in immune responses, including up-regulation of immune genes HSP70, IL-1β, transforming growth factor (TGF-β) as well as antioxidant enzymes of superoxide dismutase; catalase and glutathione peroxidase; and also a disease resistance to pathogenic agents such as *A . hydrophila* infection (He *et al*., 2011; Banerjee *et al*., 2017a; Kong *et al*., 2017).

In conclusion, Bacillus as thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant, and halophilic

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microorganisms represent unusual physiological characteristics that enable them to survive in various environmental conditions such as temperature, salinity, and pH. These bacteria are well degraders than can degrade various substrates such as cellulose, starch, proteins, agar, and hydrocarbons (Ray *et al.*, 2012). They are also nitrifiers, denitrifiers, nitrogen fixers, antibiotic producers, heterotrophic, iron precipitators, selenium oxidizers, oxidizers, and manganese reducers. In this study the in vitro potential probiotic characteristics showed that some strains of B. subtilis from trout intestine are potentially good candidate as trout probiotic. The findings of in vitro assays were confirmed by in vivo application of the B. subtilis in trout and results of growth, immune-physiological responses and resistance to *A. hydrophila* infection were promising. Further works are required to assess the bacilli efficacy in fish under different environmental conditions such as various water temperature.

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Declarations

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was approved by the Animal Use and Care Committee of Tehran University .

Conflict of Interest

The authors declare that they have no conflict of interest.

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