



Effect of *Bacillus subtilis* as a probiotic on protein, lipid content, and trypsin and chymotrypsin enzymes in rainbow trout biometry (*Oncorhynchus mykiss*)

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Abstract

This study investigated the effect of *Bacillus subtilis* IS02 as a probiotic bacterium in four different treatment conditions, namely T₁ (no probiotic), T₂ (10⁶), T₃ (10⁷), and T₄ (10⁸) cfu/g of *B. subtilis* IS02 in the diet of rainbow trout for 30 days, on the protein content, lipid content, trypsin and chymotrypsin enzyme activity, and the bioassay. The protein content and lipid content were measured using the Kjeldahl and Soxhlet methods, respectively. T₄ had the highest amount of measured protein (25.30%) while T₁ had the lowest amount (15.90). The T₄ treatment had a significant effect (*P* value = 0.001) on improving the protein content as well as the trypsin and chymotrypsin enzyme activity. In contrast, there was no significant difference in terms of the weight gain and specific growth rate between the treatments. Additionally, the results showed that the T₃ treatment had a significant effect on increasing the protein content of the carcass, although the effect was not significant in relation to the lipid content. *B. subtilis* IS02 in a concentration range of 10⁷–10⁸ cfu/g could be employed to improve the protein productive value, trypsin and chymotrypsin enzyme activity, and protein content of the carcass.

Keywords Probiotic · *Bacillus subtilis* · Trypsin · Chymotrypsin · Protein productive value

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Introduction

The increase in demand for fish seen in recent years is due to rapid population growth worldwide as well as an increased awareness of the nutritional value of fish, a preference for fish over animal proteins, and recognition that fish has both a low-calorie count and low lipid level. In order to meet the increased global demand, increasing aquaculture production, including fish, and improving the rate of production per unit area are vital considerations (Daniels et al. 2010). The use of probiotics represents a novel aquaculture technology. The probiotics typically applied in aquaculture include strains of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, *Pseudomonas*, *Rhodomonas*, *Bacillus*, *Carnobacterium*, *Lactococcus*, *Micrococcus*, *Nitrobacter*, and *rhodopseudomonas*. Probiotics are usually added to the environment and diet of breeding animals in order to enhance growth and survival rates and also help to increase digestion and the uptake of nutrition (Irianto and Austin 2002).

B. subtilis is one of the probiotics capable of properly metabolizing food due to its ability to break down proteins and carbohydrates and produces vitamins belonging to the B group, including biotin and B12 (Mombelli and Gismondo 2000).

Many studies have previously been conducted in this area. For instance, Rengpipat et al. (1998) examined the effect of the probiotic Bacillus S11 in three different treatments on Nile tilapia over 100 days. The bacterium was used in fresh cells, soluble in normal saline, and lyophilized forms. The results showed no significant difference between the three forms used in the diet, although there was a significant difference between the three treatment groups and the control group (Rengpipat et al. 1998).

In addition, Wang et al. (2008) investigated the effect of Bacillus spp. on shrimp and found significant positive effects ($P < 0.05$) on both growth rate and digestive enzymes protease and amylase between the three treatment groups examined over 28 days (Wang et al. 2008).

Farhangi and Carter (2007) evaluated the effect of probiotic bacilli on the feed efficiency and carcass quality of fingerling rainbow trout. In their study, 560 fingerling rainbow trout obtained from a breeding center. The fingerling rainbow trout were fed a diet supplemented with probiotics. The results of this study showed that the probiotics in the experimental treatment groups had a positive and significant effect on the protein efficiency ratio, lipid efficiency ratio, and protein productive value when compared to the control group ($P < 0.05$) (Farhangi and Carter 2007).

Seenivasan et al. (2012) investigated the effect of the probiotic *B. subtilis* on shrimp larvae (*M. rosenbergii*). According to their results, after 90 days, growth parameters such as the survival rate, weight gain, specific growth rate, food conversion ratio, feed efficiency, and efficiency ratio were all increased significantly in the samples fed with the probiotic ($P < 0.05$). There was also a significant increase in the amount of amino acids, carbohydrates, lipids, and ash ($P < 0.05$). The results therefore indicated that *B. subtilis*, as a probiotic, could be used to increase the survival, growth, and energy production of the shrimp (Seenivasan et al. 2012).

Merrifield et al. (2010) examined the effect of various probiotics (*B. subtilis*, *B. licheniformis*, and *Enterococcus faecium*) on the feeding of rainbow trout (*Oncorhynchus mykiss* Walbaum) previously treated with oxolinic acid. Their results showed that the use of *B. subtilis* and *B. licheniformis* in the rainbow trout feed significantly improved the food conversion ratio (FCR), specific growth rate (SGR), and protein efficiency ratio (PER). The results of this study indicated the potential role of probiotics in stabilizing and enhancing gastrointestinal microbes following treatment with antibiotics, strengthening the mechanism of

the intestinal barrier function, and protecting against potential secondary pathogens (Merrifield et al. 2010).

Therefore, the present study was conducted in order to determine the effect of *B. subtilis* IS02 at four different concentrations during 30 days of rearing on the protein content, lipid content, trypsin and chymotrypsin enzyme activity, and bioassay of rainbow trout.

Materials and methods

Preparation of the diet

The utilized water was analyzed and compared with the standard values in terms of the chlorine, pH, dissolved oxygen, total dissolved solids (TDS), total suspended solids (TSS), ammonia, nitrate, nitrite, salinity, total hardness, turbidity, conductivity, and microbial indices (Olsen 2004). Based on the recommendations of Newaj-Fyzul et al. (2007), the probiotic dosage was considered to be 10^7 cfu/g, which has previously shown the best response to the pathogenic bacterium (*Aeromonas hydrophila*) found in rainbow trout. The concentrations of 10^6 , 10^7 , and 10^8 cfu/g of the probiotic *B. subtilis* IS02 (prepared by Takgene Zist Co., Iran) were evaluated to determine the most appropriate dosage. The four chosen treatments were T₁ (10^6 cfu/g), T₂ (10^7 cfu/g), T₃ (10^8 cfu/g), and T₄ (control, no probiotic) (Newaj-Fyzul et al. 2007).

The primary probiotic powder contained 10^{11} cfu/g of *B. subtilis* IS02. As a result, concentrations of 10^6 , 10^7 , and 10^8 cfu/g were sprayed onto EXF1 feed (prepared by Kimiyagaran-e-Taghziyeh Co., Iran). After drying, soy oil was sprayed onto the food in order to prevent rapid dissolution. The food was then kept in a refrigerator until testing (Newaj-Fyzul et al. 2007).

Fish experiments

In total, 490 female rainbow trout (6.5 ± 2), whose feeding was discontinued 24 h prior to the transfer, were transferred to the hall of a fish farm located in Firuzkuh, Iran. The fish were equally divided into three bathtubs and, 48 h after the transfer, they were fed with probiotic-free foods to help them adapt to their new environmental conditions (temperature, oxygen, and pH) for 1 week. After this time, the samples were equally divided into 12 bathtubs. Subsequently, bathtubs 1 to 3 were used for treatment T₁, 4 to 6 for treatment T₂, 7 to 9 for treatment T₃, and 10 to 12 for treatment T₄. By the end of the research period, each treatment group was fed to satiation with its own special diet, and the values obtained were recorded in the respective tables.

Sampling and bioassay

Sampling was carried out using Torstensen method. During the first sampling, four fish were harvested randomly from each of the three utilized bathtubs. In order to evaluate the digestive enzymes, three random samples were selected from each replication in the second and third samplings some 30 min prior to feeding and 2 h after feeding. The samples were anesthetized using clove extract at a concentration of 100 mg/l. All biological indices were determined using the models provided by (Torstensen et al. 2008).

$$\text{SGR} = [\ln \text{ final weight} - \ln \text{ initial weight} / \text{Time}] \times 100$$

$$\text{FCR} = \text{Food consumed} / (\text{ final weight} - \text{ initial weight})$$

$$\text{PER} = \text{Weight gain} / \text{g protein intake in fish}$$

$$\text{LER} = \text{Weight gain} / \text{g lipid intake in fish}$$

$$\text{PPV} = (\text{ final protein content} - \text{ initial protein content}) / \text{protein consumed}$$

$$\text{LPV} = (\text{ final lipid content} - \text{ initial lipid content}) / \text{lipid consumed}$$

$$\text{VFI} = [\text{Food intake} / W_{\text{mean}} \times \text{Time}] \times 100$$

$$\text{FE} = \text{Weight gain} / \text{Food intake}$$

$$\text{DGI} = (\text{Weight gain} / W_{\text{mean}}) / \text{Time}$$

Preparation of the samples

The specimens were disinfected using alcohol and then placed inside the sponge that had previously been cut. A longitudinal incision was made in their abdomen. The gastrointestinal (GI) tract was completely dissected, and the specimens were washed with distilled water and then encoded. After separating the head and fins, the fish meat was also separated from the skin, packed, and transferred to a freezer at $-20\text{ }^{\circ}\text{C}$ (Kanani et al. 2014; Hosseini et al. 2017).

Preparation of the enzyme extraction

In order to prepare the enzyme extraction, a buffer containing 100 mM tris acid, 0.1 M ethylenediaminetetraacetic acid (EDTA), and 0.1% Triton X-100 with a pH of 7.8 was first prepared and then adjusted to a pH of 7.8. Each of the digestive tract samples was removed separately from the freezer and mixed with the others using a stirrer. One portion of the visceral tissue (1 g) was homogenized in nine portions of buffer (9 ml) using a homogenizer on an ice surface for 30 s. The resulting mixture was centrifuged at $20,000\times g$ for 5 min at $4\text{ }^{\circ}\text{C}$. The supernatant was then stored in a freezer ($-80\text{ }^{\circ}\text{C}$) until the pancreatic enzymes were measured (Babaei et al. 2011).

Trypsin measurement

The Bradford protein assay (1976) was used to measure both the trypsin and chymotrypsin enzymes. The trypsin was measured according to the recommendations of Erlanger et al. (1961) using 43.5 mg of a benzoyl-DL-arginine-p-nitroanilide (BAPA) substrate (Sigma Aldrich Co.) in 1 ml of dimethyl sulfoxide, which reached the final volume with a calcium chloride buffer (0.05 M tris acid, 0.02 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and a pH of 8.2). Then, 25 μl of the extracted enzyme solution was mixed with 1.25 ml of freshly prepared substrate solution and incubated for 10 min at 37 °C. The absorption rate was read at a wavelength of 410 nm using a spectrophotometer. The results were replaced in the following equation, and the relative activity of the trypsin enzyme was obtained (Erlanger et al. 1961).

$$\text{Relative activity of the enzyme trypsin} = \frac{\text{measured solution volume} \times 1000 \times \text{Sample absorbance at 410 nm}}{\text{Mg of protein in measured solution}}$$

Chymotrypsin measurement

To measure the chymotrypsin enzyme, a buffer containing 10.55 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 250 ml of 0.2 M tris(hydroxymethyl)aminomethane buffer was first prepared and adjusted to a pH of 7.8 using HCl. It was then diluted to 1 l and 432 ml of methanol was added. The 15.7 mg of substrate N-benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma Aldrich Co.) was reached in the final volume with the buffer prepared during the previous step and then incubated for 5 min at 30 °C. The absorption rate was read at a wavelength of 256 nm (Hummel 1959).

Measurement of the carcass protein and lipid content

The protein content of each rainbow trout carcass was measured using the Kjeldahl method and according to Standard 924 (AOAC 1990). Further, the lipid content was measured according to Standard 742–2003.

Data analysis methods

The results were analyzed by means of SPSS version 24 software using a univariate analysis of variance, a one-way ANOVA with the Bonferroni test, and a repeated measures ANOVA in a completely randomized design. A P value < 0.05 was considered to be significant, and the data were reported as \pm SD (standard deviation).

Results and discussion

Effect of the treatments on the growth factors

The different concentrations of the probiotic *B. subtilis* IS02 included in the diet of the rainbow trout were found to significantly increase the protein productive value (PPV). This result is similar to that of a study conducted by Farhangi and Carter (2007), who evaluated the effect of probiotic bacilli on rainbow trout fry. However, the results obtained in relation to the other

factors were different from the results of both those researchers and Wang et al. (2008), who reported a significant difference ($P < 0.05$) between the control group and the treatment groups of tilapia after 40 days of feeding with the probiotic.

There was no significant difference in terms of the other factors, including WG, SGR, FCR, PER, LER, VFI, FE, LPV, and DGI, between the control group and the treatment groups fed with different concentrations of the probiotic *B. subtilis* IS02, which is most likely due to the relatively short feeding period (30 days). In practical terms, the highest percentage of weight gain was related to treatment T₃ (225.02 ± 73.36), while the lowest percentage was seen in the control group (170.62 ± 51.17). The highest and lowest SGR values were related to treatment T₄ (3.85 ± 0.28) and the control group (3.18 ± 0.71), respectively. The highest and lowest FCR values were related to the control group (23.71 ± 9.85) and treatment T₂ (17.98 ± 8.91), respectively, while the highest and lowest protein efficiency ratios were seen for treatment T₃ (2.03 ± 0.69) and the control group (1.36 ± 0.66), respectively. In terms of the lipid efficiency ratio, the highest and lowest values were related to treatment T₃ (5.67 ± 1.92) and the control group ($1.31 \pm$), respectively. Further, the highest and lowest lipid productive values were seen for treatment T₂ (0.23 ± 0.07) and the control group (0.08 ± 0.03), respectively, while the highest and lowest voluntary feed intake values were related to the control group (98.72 ± 15.53) and treatment T₄ (63.13 ± 21.71), respectively. The highest and lowest feed efficiency values were seen for treatment T₃ (0.85 ± 0.29) and the control group (0.65 ± 0.20), respectively, while the highest and lowest daily growth index values were related to treatment T₃ (0.66 ± 0.13) and treatment T₄ (0.52 ± 0.18), respectively (Table 1). These results indicate that there was a difference between the groups. Bagheri et al. (2008) examined the effect of a probiotic on rainbow trout and observed a significant difference ($P < 0.05$) in relation to the SGR, PER, and CF factors.

Ziaei-Nejad et al. (2006) investigated the effect of a prebiotic, namely bionic yeast cell wall, on rainbow trout and reported a significant increase in the specific growth rate, body weight gain, condition factor, and protein efficiency ratio in the treatment groups fed with the bionic yeast cell wall ($P < 0.05$).

Table 1 Univariate analysis of variance analysis with Bonferroni test for rainbow trout growth in 30 days feeding with probiotic *Bacillus subtilis* IS02

T ₄	T ₃	T ₂	T ₁	
26.83 ± 219.72	225.02 ± 45.34	202.62 ± 45.34	170.62 ± 51.17	WG
0.28 ± 3.85	3.72 ± 0.85	3.61 ± 0.55	3.18 ± 0.71	SGR
1.74 ± 14.30	7.98 ± 18.88	89.17 ± 8.91	71.23 ± 9.85	FCR
0.24 ± 2	0.69 ± 2.03	1.78 ± 0.40	1.36 ± 0.66	PER
0.68 ± 5.61	1.92 ± 5.67	4.99 ± 1.12	4.36 ± 1.31	LER
21.71 ± 63.13	17.33 ± 89.6	92.81 ± 11.76	98.72 ± 15.53	VFI
0.18 ± 0.84	0.29 ± 0.85	0.75 ± 0.17	0.65 ± 0.20	FE
0.18 ± 0.52	0.13 ± 0.66	0.66 ± 0.09	0.58 ± 0.12	DGI
$0.02^{bc} \pm 0.13$	$0.01^{ab} \pm 0.04$	0.09 ± 0.01^b	0.01 ± 0.01^a	PPV
0.08 ± 0.22	0.005 ± 0.08	0.23 ± 0.07	0.08 ± 0.03	LPV

Wi/100 × Wi – Wf = WG (weight growth); SGR = $[\ln W_f - \ln W_i / T] \times 100$ (specific growth rate); FCR = $F / (W_f - W_i)$ (feed conversion ratio); PER = WG/g protein intake in fish; LER = WG/g lipid intake in fish; LPV = (final lipid content - initial lipid content) / lipid consumed (lipid productive value); PPV = (final protein content - initial protein content) / protein consumed (protein productive value); CF = $[W \times FL - 3] \times 100$; VFI = $[F / W_{\text{mean}} \times T] \times 100$ (fish value feed intake); FE = W_g / F (feed efficiency)

Merrifield et al. (2010) used *B. subtilis* and *B. licheniformis* to feed rainbow trout, and they observed a significant improvement in the FCR, SGR, and PER ($P < 0.05$) (Merrifield et al. 2010).

Signs of the same letters indicate no significant difference between the groups, $\alpha = 0.05$.

The increase in the growth factors can be attributed to the synthesis of vitamins and cofactors as well as the increased enzyme activity caused by the probiotics, which serve to improve the gastrointestinal tract and thus enhance growth (Gatesoupe 1999).

Rengpipat et al. (1998) examined the effect of Bacillus S11 on shrimp. After 100 days, their results indicated no significant difference between the groups fed with a probiotic in terms of the growth rate ($P > 0.05$), although there was a significant difference between the treatment groups and the control group (Rengpipat et al. 1998).

Naseri et al. (2013) observed positive results when using the probiotic Bioplus 2B to feed rainbow trout. In their study, the final weight, FCR, SGR, and PER all showed a significant difference between the treatment groups ($P \leq 0.05$). Meanwhile, the statistical analysis indicated no significant difference in terms of the total length and survival rate between the treatment groups ($P \geq 0.05$), while a significant difference was observed in relation to the PPV (Naseri et al. 2013).

Effect of the treatments on the digestive enzymes

A repeated measures ANOVA was used during 3 weeks of sampling (week zero, beginning of the third week, and beginning of the fifth week) to investigate the interaction of time and the probiotic concentration on the changes in the trypsin digestive enzyme. Based on the multivariate test, the lambda value was 0.045, which was significant ($P < 0.05$). According to Mauchly’s sphericity test (Table 3), the frequency of sampling is generally effective in relation to the level of trypsin, with the test values ($P = 0.269$, $X^2 = 2.62$, $W = 0.687$) showing that the main effect of time can reject the assumption of sphericity. Therefore, its corrective values ($p = 0.0$, $F = 38.71$ and $p = 0.02$, $F = 3.50$) were significant for the time variable (week) as well as the interaction between the time variable and the probiotic concentration (Table 2).

According to both Fig. 1 and Table 3, the lowest and highest trypsin activity at the beginning of the third week was related to treatment T₃ (0.025 ± 0.001) and treatment T₄ (0.051 ± 0.006), respectively.

The estimated value in the treatment T₄ group showed no significant difference to that of the treatment T₂ group (0.047 ± 0.006) ($P > 0.05$). These values at the beginning of the fifth week were related to treatment T₁ (0.017 ± 0.001), with no significant difference being seen in relation to the values for treatment T₃ (0.019 ± 0.007) and only a slight increase being seen in relation to treatment T₄ (0.052 ± 0.007), which indicates that the values were linear. In terms of the enzyme chymotrypsin, based on the multivariate test, the lambda value was 0.242, which

Table 2 Interdisciplinary effect

		Square mean	Degrees of freedom	Grade 3 sum squared	F	P
Week	Assume sprite	0.003	2	0.006	38.714	0.000
Week* concentration group	Assume sprite	0.000	6	0.002	3.501	0.021
Error (week)	Assume sprite	7.881E-5	16	0.001		

P value of first and second row shows that the effect of time and concentration has been significant ($P < 0.05$)

Estimated Marginal Means of Trypsin

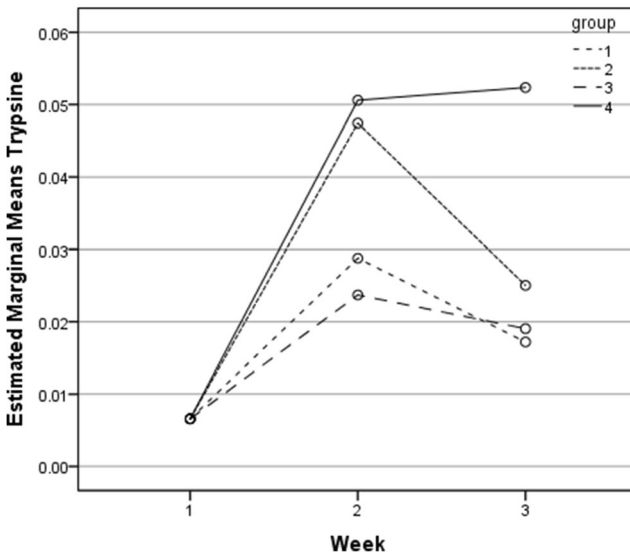


Fig. 1 Estimation of trypsin enzyme mean margin

was significant ($P < 0.05$). According to Mauchly’s sphericity test (Table 3), the frequency of sampling is generally effective in relation to the level of chymotrypsin, with the test values ($P = 0.167$, $X^2 = 3.583$, $W = 0.599$) showing that the main effect of time can reject the assumption of sphericity. Therefore, its corrective values ($P = 0.0$, $F = 14.146$ and $P = 0.0$, $F = 10.453$) were significant for the time variable (week) as well as the interaction between the time variable and the probiotic concentration (Table 4).

According to Fig. 2 and Table 5, the lowest and highest chymotrypsin activity at the beginning of the third week was related to treatment T_3 (0.552 ± 0.180) and treatment T_4 (2.436 ± 0.180), respectively. These values at the beginning of the fifth week were related to treatment T_4 (0.395 ± 0.238), with no significant difference being seen in relation to the values for treatment T_2 (0.415 ± 0.238) and only a slight increase being seen in relation to treatment

Table 3 Estimated margin values effect on the week of concentration groups

Standard error ± Mean	Week	Group
0.001 ± 0.007	1	1
0.006 ± 0.029	2	
0.007 ± 0.017	3	
0.001 ± 0.007	1	2
0.006 ± 0.047	2	
0.007 ± 0.025	3	
0.001 ± 0.007	1	3
0.006 ± 0.024	2	
0.007 ± 0.019	3	
0.001 ± 0.007	1	4
0.006 ± 0.051	2	
0.007 ± 0.052	3	

The table shows the lowest and highest amount of trypsin in samples

Table 4 Interdisciplinary effect

<i>P</i>	<i>F</i>	Square mean	Degrees of freedom	Grade 3 sum squared		
0.000	14.146	1.328	2	2.656	Assume sprite	Week
0.000	10.453	0.981	6	5.888	Assume sprite	Week* concentration group
		0.094	16	1.502	Assume sprite	Error (week)

P value of first and second row shows that the effect of time and concentration has been significant ($P < 0.05$)

T₄ (0.859 ± 0.003), which indicates that the values were linear. The digestive enzymes are among the most important factors affecting and improving the feed efficiency of fish as well as the hydrolysis of carbohydrates, lipids, and protein in the diet (Lemieux et al. 1999).

Nevertheless, only limited information is currently available regarding the extracellular enzymes secreted by the intestinal bacteria and their biochemical significance (Bairagi et al. 2002).

In the present study, the use of different probiotic concentrations of *B. subtilis* IS02 in the diet of rainbow trout was found to result in a significant difference in the level of the digestive enzymes (trypsin and chymotrypsin) between the control group and the treatment groups, which was also the case in the study by Essa et al. (2010), who investigated the effect of probiotics on the digestive enzymes (amylase, protease, and lipase) in tilapia. In our study, the highest level of trypsin was related to treatment T₄ (0.052 ± 0.006), while the lowest level was observed in treatment T₁ (0.007 ± 0.001). Additionally, the highest level of the chymotrypsin enzyme was associated with treatment T₄ (436.2 ± 0.180), while the lowest level was seen in treatment T₃ (0.555 ± 0.180) (Essa et al. 2010).

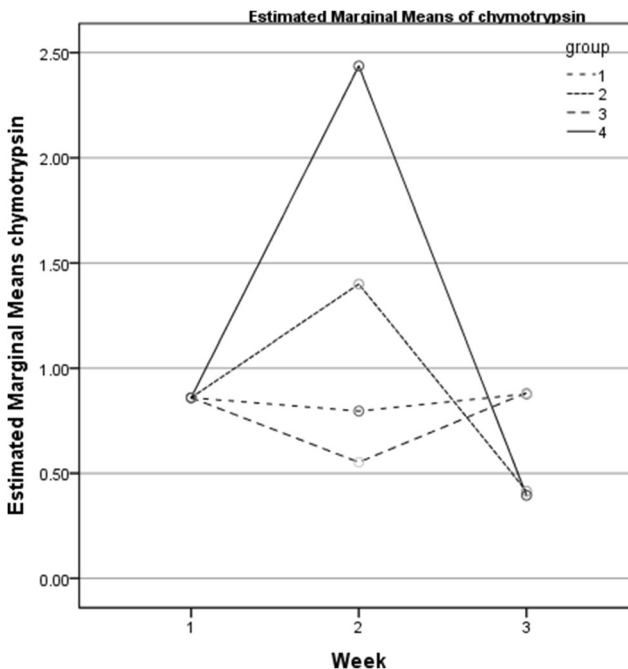


Fig. 2 Estimation of chymotrypsin enzyme mean margin

Table 5 Estimated margin values effect on the week of concentration groups

Standard error ± Mean	Week	Group
0.003 ± 0.859	1	1
0.180 ± 0.796	2	
0.238 ± 0.878	3	
0.003 ± 0.859	1	2
0.180 ± 1.400	2	
0.238 ± 0.415	3	
0.003 ± 0.859	1	3
0.180 ± 0.552	2	
0.238 ± 0.883	3	
0.003 ± 0.859	1	4
0.180 ± 2.436	2	
0.238 ± 0.395	3	

Adding probiotics as a living supplement to the diet can help them to become established in the GI tract (Fuller and Perdigon 2003). These microorganisms as well as the enzymes secreted by them play an important role in the digestive process (Munilla-Moran et al. 1990).

Further, they stimulate the production of intracellular enzymes (Ziaei-Nejad et al. 2006) and thus increase digestibility. The increase in the digestive enzymes seen in this study may be due to the probiotic activity of *B. subtilis* IS02 and the production of the associated extracellular enzymes. However, it is difficult to distinguish between the intracellular enzymes produced by the fish and the extracellular enzymes produced by the probiotics in the GI tract (Essa et al. 2010).

The effect of the treatments on the carcass protein and lipid content

The sampling was performed at the end of the study period in order to compare the protein and lipid content in the various concentrations of the probiotic *B. subtilis* IS02. The changes were studied using a one-way analysis of variance with the Bonferroni test. In terms of the protein content, according to Table 6, the various concentrations of the probiotic *B. subtilis* IS02 appear to have had different effects on the protein content of the rainbow trout during the 30-day research period ($p = 0.001$). The significance limit of the ANOVA for the lipid content was greater than 0.05 ($p = 0.414$), which indicates that the different concentrations of the probiotic *B. subtilis* IS02 had no effect on the variation in the lipid content seen in the rainbow trout (Table 6).

Naseri et al. (2013) observed positive results in relation to the use of the probiotic Bioplus 2B in rainbow trout, so that there was a significant difference in the protein and lipid content of the carcass between the different treatment groups at the end of the experimental period ($P < 0.05$). The results of using different concentrations of the probiotic *B. subtilis* IS02 in the diet of rainbow trout indicated an increase in the crude protein content when analyzing the composition of the carcass. This result is similar to the findings obtained by (Farhangi and Carter 2007) and Azevedo et al. (2012) in relation to the effect of *B. subtilis* and mannan-oligosaccharide on tilapia, which is likely due to the production of protease enzymes in the GI tract (Irianto and Austin 2002). This serves to improve both digestion and the uptake of protein compounds (Fuller and Perdigon 2003). The findings of the study conducted by El-Haroun et al. (2006) are also consistent with the results of the abovementioned research, showing a significant difference in the protein content of tilapia under the influence of probiotics ($P \leq 0.01$) (El-Haroun et al. 2006).

Table 6 Changes in protein and fat levels of rainbow trout in four groups fed with probiotic levels of *Bacillus subtilis* IS02

	Groups	Number	Limit of significance	Std. Error	mean
Protein content	1	9	0.000	0.07	3.64 ^α
	2	9		0.04	3.62 ^b
	3	9		0.16	4.06 ^a ^b
	4	9		0.05	3.58 ^b ^c
Lipid content	1	9	0.14	0.06	2.34
	2	9		0.07	2.37
	3	9		0.06	2.62
	4	9		0.06	2.34

Signs of the same letters in the mean columns indicate a significant difference between the groups, $\alpha = 0.05$

The change in the carcass lipid content was not significant in the different treatment groups, which is consistent with the findings of Daniels et al. (2010) in relation to the effect of *B. subtilis*, *B. licheniformis*, and *E. faecium* on rainbow trout as well as those of Ghaljaei et al. (2016), although it differs from the results of Farhangi and Carter (2007), who found a significant difference between the treatment groups ($P < 0.05$). The changes in the lipid and protein content could be related to the changes in their synthesis and storage in the fish muscles (Abdel-Tawwab et al. 2005).

Conclusion

The aim of this study was to investigate the effects of different concentrations of the probiotic *Bacillus subtilis* IS02 on the protein content and digestive enzymes of rainbow trout using a spectrophotometer and a bioassay. In order to determine the proper dosage of the probiotic *B. subtilis* IS02 as well as its effect on the protein content, growth, and trypsin and chymotrypsin enzymes in rainbow trout, four treatment groups were created, namely T₁ (10⁶ cfu/g), T₂ (10⁷ cfu/g), T₃ (10⁸ cfu/g), and T₄ (control, no probiotic). Finally, based on the results of the bioassay and the measurement of the digestive enzymes, proteins, and lipids in the composition of the carcass, the dose that had the best effect on the rainbow trout was selected as the optimal dosage of the probiotic *B. subtilis* IS02. The results concerning the PPV factor indicated that the different concentrations of the probiotic *B. subtilis* IS02 had various effects on the rainbow trout during the 30-day study period when compared to the control treatment ($p = 0.001$). There was also a significant difference between the groups in terms of the trypsin and chymotrypsin enzyme activity ($p = 0.05$). Additionally, the effects of the probiotic concentrations on the protein content of the rainbow trout differed during the 30-day study period ($P = 0.001$). The significance limit of the ANOVA in relation to the lipid content was greater than 0.05 ($P = 0.414$), which indicates that the different concentrations of the probiotic *B. subtilis* IS02 had no effect on the variation in the lipid content seen in the rainbow trout (Table 4). The present findings demonstrated that a concentration of 10⁸ cfu/g is most suitable for use in the diet of rainbow trout due to the positive effects on the trypsin and chymotrypsin enzyme activity as well as the protein content seen in treatment T₄. In this regard, 10⁷ cfu/g of the probiotic *B. subtilis* IS02 also had the best effect on the carcass protein content of the rainbow trout in treatment T₃. Hence, it can be concluded that the most appropriate concentration of *B. subtilis* IS02 for use in the diet of rainbow trout is within the range of 10⁷ to

10^8 cfu/g, which leads to the stimulation of the digestive enzymes, the elevation of the carcass protein content, and, consequently, the high-feed efficiency of the rainbow trout.

Compliance with ethical standards

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

Ethical approval This article does not contain any studies with animals performed by any of the authors.

References

- Abdel-Tawwab M, Mousa MA, Sharaf SM, Ahmad MH (2005) Effect of crowding stress on some physiological functions of Nile tilapia, *Oreochromis niloticus* (L.) fed different dietary protein levels. *International Journal of Zoological Research* 1:41–47
- AOAC (1990). Official Methods of Analysis. Washington, DC: Association of Official Analytical Chemists
- Azevedo AS, Sousa S, Jacinto A (2012) An amputation resets positional information to a proximal identity in the regenerating zebrafish caudal fin. *BMC Dev Biol* 12:24
- Babaei SS, Kenari AA, Nazari R, Gisbert E (2011) Developmental changes of digestive enzymes in Persian sturgeon (*Acipenser persicus*) during larval ontogeny. *Aquaculture* 318:138–144
- Bagheri T, Hedayati SA, Yavari V, Alizade M, Farzanfar A (2008) Growth, survival and gut microbial load of rainbow trout (*Oncorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. *Turk J Fish Aquat Sci* 8:43–48
- Bairagi A, Ghosh KS, Sen SK, Ray AK (2002) Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquac Int* 10:109–121
- Daniels CL, Merrifield DL, Boothroyd DP, Davies SJ, Factor JR, Arnold KE (2010) Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture* 304:49–57
- El-Haroun E, Goda AS, Chowdhury K (2006) Effect of dietary probiotic Biogen® supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquac Res* 37:1473–1480
- Erlanger BF, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Arch Biochem Biophys* 95:271–278
- Essa MA, El-Serafy S, El-Ezabi MM, Daboor SM, Esmael NA, Lall SP (2010) Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia, *Oreochromis niloticus*. *Int J Zoo Res* 5:143–162
- Farhangi M, Carter CG (2007) Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac Res* 38:1274–1282
- Fuller R, Perdigon G (2003) Gut flora, immunity and health. Blackwell Publishing, Hoboken
- Gatesoupe F (1999) The use of probiotics in aquaculture. *Aquaculture* 180:147–165
- Ghaljaei FA, Khara H, Shenavar MA (2016) Effect of lactobacillus plantarum (kc426951) bacteria isolated from the intestine of rainbow trout guilan on hematological indices and immune of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) The quarterly journal of animal physiology and development (quarterly journal of biological sciences) winter 2016, 9(32):61-68
- Hosseini R, Ahari H, Mahasti P, Paidari S (2017) Measuring the migration of silver from silver nanocomposite polyethylene packaging based on (TiO₂) into *Penaeus semisulcatus* using titration comparison with migration methods. *Fish Sci* 83(4):649–659
- Hummel BC (1959) A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can J Biochem Physiol* 37:1393–1399
- Irianto A, Austin B (2002) Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 25:333–342
- Kanani HG, Nobahar Z, Kakoolaki S, Jafarian, H (2014) Effect of ginger-and garlic-supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiol Biochem* 40(2):481–490

- Lemieux H, Blier P, Dutil J-D (1999) Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? *Fish Physiol Biochem* 20:293–303
- Merrifield D, Bradley G, Baker R, Davies S (2010) Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquac Nutr* 16:496–503
- Mombelli B, Gismondo MR (2000) The use of probiotics in medical practice. *Int J Antimicrob Agents* 16:531–536
- Munilla-Moran R, Stark J, Barbour A (1990) The role of exogenous enzymes in digestion in cultured turbot larvae (*Scophthalmus maximus* L.). *Aquaculture* 88:337–350
- Naseri S, Khara H, Shakoori M (2013) Effects of probiotics and Fe ion on the growth and survival and body composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) fry. *J Appl Anim Res* 41:318–325
- Newaj-Fyzul A, Adesiyun AA, Mutani A, Ramsubhag A, Brunt J, Austin B (2007) *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J Appl Microbiol* 103:1699–1706
- Olsen Y (2004) Live food technology of cold-water marine fish larvae. *Culture of cold-water Marine Fish* 73–128
- Rengpipat S, Phianphak W, Piyatiratitivorakul S, Menasveta P (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167:301–313
- Seenivasan C, Radhakrishnan S, Muralisankar T, Bhavan PS (2012) *Bacillus subtilis* on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *Macrobrachium rosenbergii* post larvae. *Egypt J Aquat Res* 38:195–203
- Torstensen B, Espe M, Sanden M, Stubhaug I, Waagbø R, Hemre G-I, Fontanillas R, Nordgarden U, Hevrøy E, Olsvik P (2008) Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. *Aquaculture* 285:193–200
- Wang Y-B, Tian Z-Q, Yao J-T, Li W-F (2008) Effect of probiotics, *Enterococcus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture* 277:203–207
- Ziaei-Nejad S, Rezaei MH, Takami GA, Lovett DL, Mirvaghefi A-R, Shakouri M (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Penaeus indicus*. *Aquaculture* 252:516–524