Research Article

Effects of diet supplementation with different level of Celmanax® (*Saccharomyces cerevisiae* cell wall with Mannan-Oligosaccharides) on health, environmental stress and Yersiniosis in *Oncorhynchus mykiss*

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Abstract

This study aimed to investigate the effects of complementary rainbow trout diets with different concentrations of Celmanax® (active compounds of *Saccharomyces cerevisiae* with mannan-oligosaccharide (MOS)) on immune responses, pressure resistance and resistance to Yersiniosis. Rainbow trout were fed with a diet containing various concentration levels of *S.cerevisiae* with MOS (prebiotic) (0, 0.1, 0.5 and 1%) for 60 days. While evaluating some of the parameters of the immune system, the blood samples were prepared from the tuber stem vein every 30 days. On day sixty of the study, various stress tests including temperature increases, hypoxia and induction of experimental disease with *Yersinia ruckeri* were also performed in all the experimental groups. Results showed that lysozyme activity, alternative complement pathway and total antibody were significantly elevated by diets containing different concentrations of prebiotic and the effective supplementation diet concentration was found to be 0.1% ($p<0.05$). However, the results of environmental pressures and exposure to bacteria showed that rainbow trout resistance was increased with different concentrations of prebiotic and the effective supplementation diet concentration was also 0.1% ($p<0.05$). Based on these findings, it is suggested that to increase and enhance immunity and improve rainbow trout resistance, it would be appropriate to add 0.1% concentration of prebiotic in the diet.

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Introduction

Bacterial diseases in freshwater fish are among the most commonly found diseases in aquaculture although some of them are preventable. Environmental disorders such as poor water quality, oxygen deficiency and temperature changes are among the risk factors of bacterial diseases. One of the most common diseases of the aquaculture industry in Iran is Yersiniosis, which annually causes huge economic losses to the country's aquaculture industry. In Iran, for the past few years the aquaculture production has witnessed a gradual down fall due to outbreak of bacterial infections like Enteric Red mouth Disease (ERMD). To increase the nutrient conversion rate, enhance the immune responses, increase the resistance to environmental stress and other diseases, identification of new methods without antibiotic administration, is essentially needed in farmed fish. Previous reports have indicated that the use of prebiotic in aquatic animal's increases parameters such as survival rate, feed conversion ratio, food digestibility, digestive activities, enzyme activities, immune system, ultimately the intestinal bacterial population and contributes to the prevention of pathogens, especially bacterial diseases (Dimitroglou *et al.,* 2011a). The first aim of this study was to investigate the effect of various concentration levels of Celmanax® supplementation on the reduction of farmed rainbow trout mortality in an experimental challenge with Yersiniosis. Many studies by several research groups have been carried out and highlighted the effects of prebiotic in various species of fish including Salmonidae family, Cyprinidae, Tilapia (Burr *et al*., 2005; Gatlin III *et al*., 2006; Denev *et al*., 2009; Yousefian and Amiri, 2009; Merrifield *et al*., 2010; Ringø *et al*., 2010; Sweetman *et al*., 2010 and Dimitroglou *et al*., 2011a). Reports are indicating the beneficial effects of commonly used prebiotic in aquatic animals such as inulin, fructoiligosaccharides, short-chain fructo-oligosaccharides, oligophroctoses, mannan-oligosaccharides, transgenic galactulosaccharides, galactosaligosaccharides, xylovalciosaccharides, arabin oligosaccharides, isomaltoligosaccharides and other commercial products (Pryor *et al*., 2003; Genc *et al.,* 2007;; Dimitroglou *et al*., 2009; 2010a; 2010b; Ringø *et al*, 2010; Sweetman *et al*., 2010; Dimitroglou *et al*., 2011a; Dimitroglou *et al*., 2011b; Dimitroglou *et al*., 2011c). The importance of prebiotics in some species of the Salmonidae family such as Atlantic salmon has been already highlighted. The accessibility of different prebiotics and the successful use of prebiotic in human beings and other animals has made it easier to select prebiotics in the aquatic industry (Azad and Al-Marzouk, 2008). An important matter in the selection of known prebiotics is the variation in aquatic organisms (due to environmental conditions and hostile differences) compared to other organisms, and this difference may lead to some inefficiency. Specific differences resulted in the use of microflora and aquatic organism probiotics rather than other probiotics (Merrifield and Ringo, 2014).

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In the aquaculture industry and in particular, in large-scale cold water fish production, diseases and stress often occur and cause huge economic losses. Probiotics and prebiotics are potential alternatives, which are naturally built to protect the host from bacterial pathogen and do not have environmental problems (Markowiak and Slizewska, 2017). The use of Probiotics and Prebiotic have some benefits such as inhibiting the expression of acute genes, increasing enzymes secretion, and ultimately improving the growth parameters and immune responses and declining the environmental pressure (Merrifield and Ringo, 2014). The presence of various diseases in trout fishes is considered as a limiting factor in production, and some old strategies such as using chemicals for prevention and treatment as well as vaccination due to the emergence of resistant bacteria to various antibiotics are obsolete. (Balcázar *et al*., 2006; Cabello, 2006; Romero *et al*., 2012; Pérez-Sánchez *et al*., 2013). Today, overuse and misuse of antibiotics have led to a rise in antibiotic resistance. Antibiotic resistance occurs when bacteria are no longer sensitive to a medication that should eliminate an infection. Antibiotic-resistant bacterial infections are potentially very dangerous and increase the risk of death. Among other prebiotics, Mannan-oligosaccharides containing diets affected the microbiota and increased the diversity of microflora (Dimitroglou *et al*., 2010a; Torrecillas *et al*., 2011; 2014). However, no significant modulation effect was observed when Celmanax® (contained active compounds of *S. cerevisiae* with MOS, as a probiotic with other Mineral and amino acids) was added to the rainbow trout diet, suggesting that any potential effect was masked by the greater general effect of dietary rainbow trout on the GUT microbiota and immunity. In the current study, we aimed to evaluate the effects of supplemented diet with different concentrations of Celmanax® (Vi-COR®, Mason City, IA, USA) as a prebiotic (Kaur and Bansal, 2006) on the immunity, resistance against environmental pressures, Yersiniosis, stress indices (plasma glucose, cortisol levels) and experimental challenges in rainbow trout.

Materials and methods

The present investigation has been under taken for a period of three years from November 2015 to September 2017. In this study, 600 rainbow trout fishes with an average weight of 19.08 ± 1.45 grams were transferred to Artemia and Aquaculture research institute, Urmia University after obtaining health certificates from the reference laboratory of the Iranian Veterinary Organization. The fishes were disinfected with saline (10 g/lit) at first (Tukmechi *et al*., 2011; Tukmechi and Bandboni, 2013). They were kept for 7 days in 1000 liters of fiberglass basins to adapt to laboratory conditions. Fish breeding and nutrition were carried out with different concentrations of prebiotic in 300 liters of fiberglass ponds with a density of 50 fishes per tank for 60 days. Water was supplied through a semi-deep well and during the growing period, the physicochemical parameters of water were measured and recorded daily. In

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this study, the solution preparation of Celmanax® was prepared. The product contains at least 30% crude protein, 4% crude fat, 12% moisture and 1% fiber. The feeding protocol for fishes was based on the percentage of live weight of fish and water temperature by using the commercial grade of the platform.

The fishes in the first group (control group) were fed only with commercial foods without the addition of any prebiotics and the other treatment groups were treated with various concentrations of Celmanax® (0.1, 0.5 and 1%). To add the prebiotic, first, the amount of daily food in each group was calculated, and then the required amount of prebiotic was sprayed on all parts of the food and dried for 2 hours at room temperature and the clean place (Tukmechi *et al*., 2011). Food preparation was performed daily. Different treated groups were fed using prebiotic during 60 days of the experiment. At the end of the experiment period (60 days), to evaluate prebiotic effects on immunological parameters, all treated groups were fed standard food for 15 days (Tukmechi *et al*., 2011; Gharekhani *et al*., 2015).

Intestines microflora

The intestinal contents were extracted by dissecting the fish, removing the intestine and squeezing out the contents for microbial analysis. Samples were taken to measure the yeast and bacteria in the contents of intestinal fish of different groups on days 0, 30, 60. To perform a bacterial total count from intestinal contents, serial dilutions (10^{-1}-10^{-8}) were prepared in sterile normal saline (pH 7.2), and thereafter 100 µl from each dilution was cultivated in McConkey agar, Trypticase Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) mediums and incubated for 48 hours at 20-25 °C. After incubation, the growing colonies were counted and the total number of colonies was determined and finally expressed as Colony Forming Unit (*CFU*) per gram of intestinal contents (Zhou *et al*., 2009; Tukmechi *et al*., 2011).

Immunological parameters

The intrinsic immune parameters including lysozyme activities, alternative complement pathways, and total antibody levels were measured to evaluate the immune responses of rainbow trout fed with different concentrations of prebiotics. Sampling was carried out on days 0, 30, 60 and 75. Three pieces of fish (nine pieces per treatment) were randomly selected. Fishes were anesthetized with 200 mg/l clove oil, and then blood samples were collected from a cardinal vein using a heparin-coated syringe and transferred into sterile tubes. The blood samples were allowed to clot at room temperature and stored in a refrigerator overnight. The clot was then centrifuged at 1500 × g for 5 min. Then the serum was collected and stored in sterile Eppendorf tubes at -20 °C until further immunological analyses (Tukmechi *et al*., 2011; Tukmechi and Bandboni, 2013).

Lysozyme activity in serum

The lysozyme activity of serum was measured according to the previously described method (Demers and Bayne., 1997). This assay works

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based on the lysis of the lysozyme-sensitive Gram-positive bacterium, *Micrococcus lysodiekticus* (Sigma, USA). Lysozyme acts upon susceptible bacteria by combining with and breaking down a mucopolysaccharide, which is located in the bacterial cell wall. *M. lysodeikticus*, one of the Gram-positive bacteria, is normally highly sensitive to lysozyme. Three dilutions of hen's egg white lysozyme (Sigma) ranging from 0 to 25 µg /ml (in 0.1 M phosphate-citrate buffer, pH 6, Sigma, USA) were used as the standard. Prepared standard solutions were placed along with the undiluted serum sample (25 µl) in the wells of a 96-well plate in triplicate, 175 µl of *M. lysodiekticus* suspension (750 µg/ml) was prepared in the same buffer then added to each well. After vigorous mixing, the change in turbidity was measured on 0 and 4 min at 450 nm at approximately 20°C using a microplate reader (Statt facts, Germany). The equivalent unit of activity of the sample as compared to the standard was determined and expressed as µg/ml serum.

Haemolytic serum complementary activity (ACH50)

The haemolytic complement activity was assayed using rabbit red blood cells (RaRBC) as targets (Amar *et al*., 2000). Rabbit red blood cells (RaRBC) were washed three times in ethylene glycol tetra-acetic acid-magnesium-gelatinveronal buffer (0.01 M EGTA-MgeGVB, pH 7) and the cell numbers were adjusted to 2×10^8 cells /ml in the same buffer. At first, 100% lysis value was obtained by adding 100 µl of the above RaRBC to 3.4 ml distilled water. The hemolysate was centrifuged (5 min × 400g) and the optical density (O.D.) of the supernatant was determined at 414 nm using a spectrophotometer (Awareness, USA). Following this, the test sera were diluted (100 times), different volumes ranging from 100 to 250 µl (total volume was adjusted to 250 µl with the buffer) were allowed to react with 100 µl of RaRBC in test tubes. This mixture was incubated at 20°C for 90 min with intermittent mixing, following which 3.15 ml of 0.85% NaCl solution was added and the tubes were centrifuged at 1600 × g for10 min at 4 °C and the O.D. of the supernatant was measured as mentioned above. A lysis curve was obtained by plotting the percentage of haemolytic against the volume of serum added on a log-log graph. The volume yielding 50% haemolytic was used for determining the complementary activity of the sample as follows:

ACH50 (units/ml) = K × (reciprocal of the serum dilution) ×0.5

Where K: The amount of serum (ml) giving 50% lysis; 0.5: The correction factor since the assay was performed on half-scale of the original method.

Total antibody levels of serum

Total serum immunoglobulin is determined following the method of Siwicki and Studnicka (1994). After dilution of serum samples with 0.85% NaCl (half-fold), total protein content is determined by the Bradford method. Briefly, 100 µl of serum samples were mixed with an equal volume of 12% solution of polyethylene glycol (Sigma, USA) in wells of a 96-well microliter plate. After 2 hours of incubation at room temperature, the plate was

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centrifuged at 500×g at 4°C. The supernatant has diluted half fold with 0.85% of NaCl and the protein content was determined by the Bradford method. This value was subtracted from the total protein level and the result was equal to the total immunoglobulin concentration of the serum that was expressed as mg/ml.

Hypoxia and high-temperature stress

Fifteen fishes were harvested randomly from each experimental group in deficit pressure of oxygen (five fishes per replicate) and transferred to 100 liter basins. The oxygen levels of the ponds were directly reduced to 3 ppm and the fishes were kept for 24 hours in this condition. Following the aforementioned stress and after half an hour, blood samples were collected from all groups to measure blood cortisol and glucose levels (Gharekhani *et al.,* 2015). For high-temperature stress, 15 fishes from each treatment (five fishes per replicate) were randomly caught and transferred to a 100-liter pond, which was divided into two equal parts. The water temperature of the pond was increased to 26 °C for 30 to 45 minutes by aquarium heater, allowing the fish to stay in this condition for 24 hours. Also, after 90 min of simultaneous pressure on all groups, the blood sample was taken to measure the cortisol and the blood glucose levels using the standard method. In addition, physicochemical parameters of water and death rates recorded during either stress (Tukmechi *et al.*, 2011; Tukmechi and Bandboni, 2013). After recording the mortality rate, the survival rate formula (number of deaths - initial number/initial number × 100) was used to determine the survival rate.

In situ *Y. ruckeri* challenge

After a 60-day feeding trial, 30 fish from each experimental group (10 fish per tank) were randomly chosen, anaesthetized with clove powder (200 mg/l). Then, the fish were challenged by I.P. injection of 0.1 ml from *Y. ruckeri* (BCCM/LMG 3279; Belgium, Co-Ordinated Collection of Microorganisms) suspension (1.1×10^7 *CFU*/ml). Dead and moribund fish were removed and examined microbiologically for up to 14 days. Moreover, an agglutination test was performed on samples to confirm the infection caused by *Y. ruckeri* (Tukmechi and Bandboni, 2013).

Statistical analysis

The data (Mean ± standard deviation) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test to compare the means between individual treatments by using SPSS (Version 21; SPSS Inc.,) at $p < 0.05$ level.

Results

Celmanax® elevated bacteria and yeast populations in the intestine

Data gathered from water physicochemical indicators showed that there was no significant difference in terms of physicochemical indices of water in rainbow trout's during the breeding and experiment period. Also, the values of all indices were within the normal range for the production of rainbow trout. The results showed the intestinal microflora in Celmanax-received groups was significantly different from the control group ($p<0.05$). The highest population of microflora was counted on day 30 in the group of fish that received 1%

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concentration of Celmanax® (Table 1). The above obtained results were also the same after 60 days Celmanax® administration. The population of yeast in the digestive tract was also recorded (Table 2). We found that the GUT yeast population was increased in a time- and concentration-dependent manner as with increasing of Celmanax® consuming time and concentration, the density of yeast has been elevated when compared with those fish which nominated as the control group. The highest population of yeasts (5.43×10^4) was found in the intestine of fishes, which received 1% concentration of prebiotic.

Table 1. Bacteria density in the total count of intestine content in rainbow trout's fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9)

Treatment day	Control	T1	T2	T3
Day 0	$5.77\times10^{4\ a}$	$5.34\times10^{4\ a}$	$5.23\times10^{4\ a}$	$5.44\times10^{4\ a}$
Day 30	$5.75\times10^{5\ d}$	$4.29\times10^{6\ c}$	$5.03\times10^{6\ b}$	$9.58\times10^{6\ a}$
Day 60	$5.84\times10^{5\ d}$	$1.24\times10^{7\ c}$	$2.79\times10^{7\ b}$	$7.10\times10^{8\ a}$

The various superscripts in the same row indicate a significantly different ($p<0.05$).
(T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Table 2. Yeast density in the total count of intestine in rainbow trout's fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9)

Treatment day	Control	T1	T2	T3
Day 0	105^{a}	95^{a}	89^{a}	93^{a}
Day 30	121^{b}	$2.5\times10^{3\ a}$	$3.1\times10^{3\ a}$	$3.12\times10^{3\ a}$
Day 60	130^{c}	$3.1\times10^{3\ b}$	$3.4\times10^{3\ b}$	$5.43\times10^{4\ a}$

The various superscripts in the same row indicate a significantly different ($p<0.05$).
(T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

The lysozymal activity of serum was enhanced in Celmanax-received fish

The results of lysozyme activity measurements in serum are presented in Figure 1. We found that the lysosomal activity elevation was not concentration-dependent as the maximum activity was measured in those fish that were fed a diet with 0.1 % Celmanax®. On day 75 of the study, with the discontinuation of prebiotic in the diet for 15 days, lysozyme enzyme activity significantly decreased in all prebiotic fed groups ($p<0.05$).

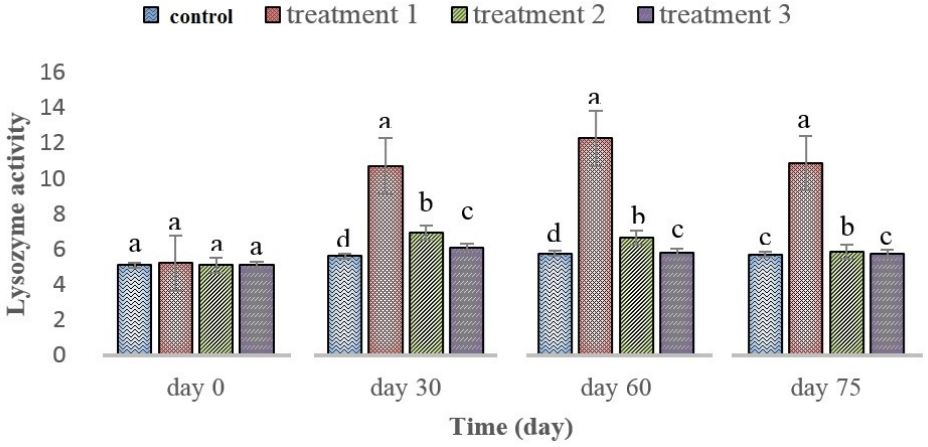

Figure 1. The lysozyme activity of serum in rainbow trout fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9). The various superscripts in the same row indicate a significantly different ($p<0.05$). (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

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The haemolytic complementary activity was elevated by Celmanax® supplementation

The haemolytic complement activity of serum from different experimental groups and in various supplementation times are presented in Figure 2. On day 30, fishes were treated with different concentrations of prebiotic showed a significant ($p<0.05$) increase of haemolytic complementary activity when compared to the control group. The lowest concentration (0.1%) of prebiotic resulted in a remarkable elevation of complementary activity on days 30 and 60. The complementary activity elevation was found not to be concentration-dependent as we found the highest increase in fishes, which received only 0.1% of the Celmanax®. On day 75 of the study, with the discontinuation of prebiotic in the diet for 15 days, the haemolytic complement activity decreased significantly in all prebiotic received groups ($p<0.05$).

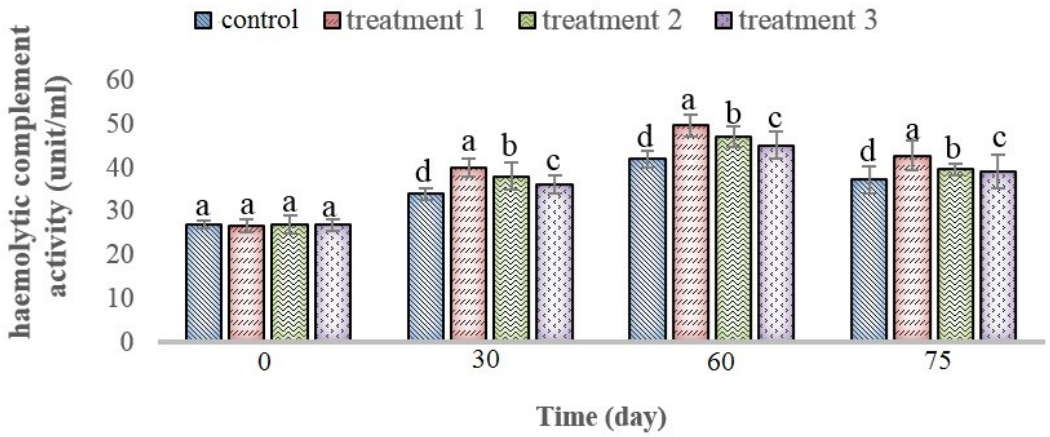

Figure 2. The haemolytic complement activity in rainbow trout's fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9). The various superscripts in the same row indicate a significantly different ($p<0.05$). (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Total antibody level was increased in prebiotic-received fish

The total level of antibody in sera from fishes fed with Celmanax® prebiotic or diet without any extra probiotics are shown in Figure 3. No significant differences were observed on day zero ($p>0.05$) in terms of total serum antibody levels. Comparing the prebiotic-received fishes with those not received, indicated that albeit with minor differences but Celmanax®-received fish showed a statistically higher level of antibody. The highest level of antibody was found in the serum of those fish fed the diet containing 0.1 % Celmanax® and fed that diet for 60 days. On day 75 of the study, with the discontinuation of prebiotic administration for 15 days, total serum antibody levels decreased significantly in all test groups ($p<0.05$).

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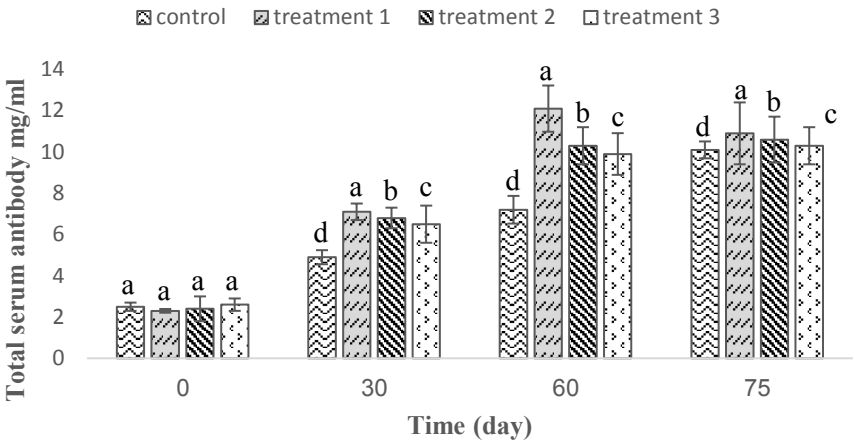
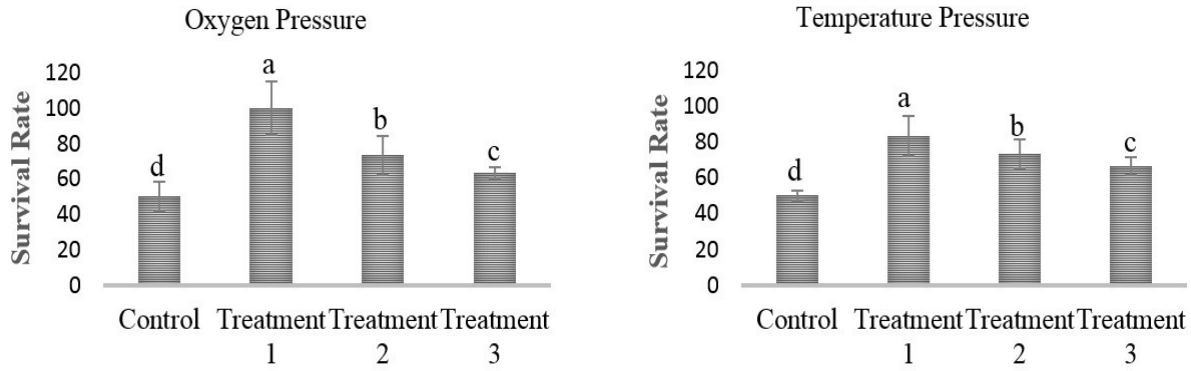


Figure 3. The total serum antibody in rainbow trout fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9). The various superscripts in the same row indicate a significantly different ($p<0.05$). (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Celmanax® enhanced the resistance of fish to oxygen deficiency and temperature changes

The prebiotic supplementation resulted in a remarkable and concentration-dependent elevation of survival index when the fish encountered oxygen stress (Figure 4). The average death time (50% mortality) was 3 hours and 50 minutes. The lowest death rate was found in the group of fish that fed the lowest given concentration of Celmanax®. We found the same profile of survival index when fish were exposed to abnormal temperature. The obtained results showed that the death rate declined in the Celmanax®-containing diet fed fish in a concentration-dependent manner. Although a prebiotic-containing diet could prevent totally (100%) from abnormal oxygen-dependent death, high temperature-induced death rate only could be restricted by 83% (Figure 5).



Figures 4-5. The resistance of fish to oxygen deficiency and temperature changes and survival rate of rainbow trout fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9). The various superscripts in the same row indicate a significantly different ($p<0.05$). (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

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Measurement of plasma glucose and cortisol levels

The results of measuring plasma glucose and cortisol serum after 30 min of exposure to hypoxia and/or high temperature are presented in Table 3. The results showed that in fishes fed with different concentrations of prebiotic, levels of cortisol and plasma glucose after high-temperature stress were significantly ($p<0.05$) reduced. In both hypoxia and temperature stress the lowest levels of serum cortisol and plasma glucose were observed in the group, fed with 0.1% Celmanax® prebiotic concentration.

Table 3. Cortisol and glucose levels in rainbow trout's fed with Celmanax® prebiotic supplemented diets. Values are presented as mean ± SD (n=9)

	Oxygen stress		Temperature stress	
	plasma glucose (mg/dl)	**Cortisol (µg/dl)**	**plasma glucose (mg/dl)**	**Cortisol (µg/dl)**
Control	256±28 [a]	2.693±0.05 [a]	280±4.12 [a]	2.710±0.1 [a]
Treatment 1	93±3 [d]	1.030±0.05 [d]	117±24 [d]	0.802±0.15 [d]
Treatment 2	122±21 [c]	1.332±0.1 [c]	198±19 [c]	1.016±0.2 [c]
Treatment 3	228±14 [b]	1.441±0.1 [b]	244±5.3 [b]	1.030±0.05 [b]

The various superscripts in the same row for each title of the study indicate a significantly different ($p<0.05$).
(T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

Celmanax elevated the survival rate in experimentally-induced Yersiniosis

The results of death rate in the fishes, which exposed against the bacterium causing Yersiniosis for two weeks, revealed that all three given concentrations of prebiotic declined the death rate when compared to the control group. The most survival rate (73.33 ± 5.77%) was observed in fish fed with 0.1% Celmanax® (Figure 6).

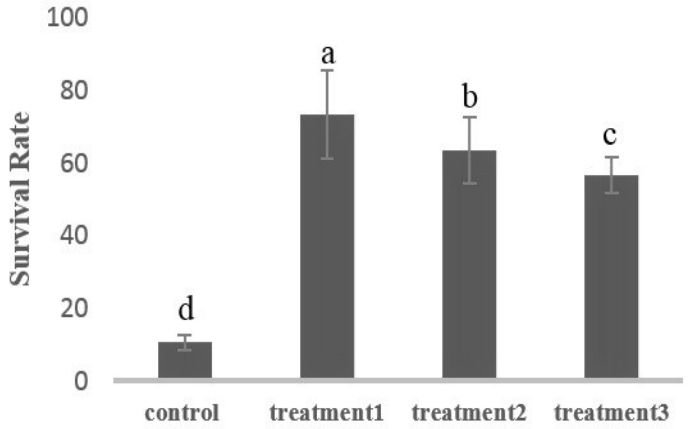

Figure 6. Percentage of survival rate of rainbow trout fed with Celmanax® prebiotic supplemented diets after experimental challenge with *Y. ruckeri*. Values are presented as mean ± SD (n=9). The various superscripts in the same row indicate a significantly different ($p<0.05$). (T1: 0.1, T2: 0.5 and T3: 1 percent of prebiotic in commercial pellet).

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Discussion

Breeding fish are always important sources of protein in the world. The presence of various diseases, such as bacterial diseases is one of the important problems in intensive fish culture. The idea to use *S. cerevisiae* and MOS in rainbow trout feeds related to the concept that certain sugars, particularly mannose, could be used to largely block the colonization of intestinal pathogens such as *Y. ruckeri*. When they bind to the MOS product, the pathogens are prevented from attaching to intestinal mannose, proliferating, and producing toxins. A second reason for developing the MOS product was because of their effectiveness on some strains of live yeast in binding and reducing intestinal pathogen counts. Several yeast companies now are manufacturing yeast cell wall products containing MOS (Benites *et al.*, 2008).

The first item which has been analysed in the current study was the population of GUT microflora. The findings of the study revealed that prebiotic-received fish had a remarkably higher number of microbiota. It has been reported that there are several issues concerning the GUT microbiota including the diversity of strains in various animals and even between fish species, differences between the number of microflora population among the fish species and also the difference in the members of microbiota forming organisms (Austin, 2006). The significance of rich GUT microbiota could be reflected in the degradation of various energy resources and providing other essential compounds due to their activities including vitamins and other nutrients. Therefore, our results may suggest that using Celmanax® in proper concentration as a prebiotic might help the fishery industry to have a higher production capacity. Our findings in this study are supported by previous reports (Dimitroglou *et al.,* 2009; Azari *et al.,* 2011; Ortiz *et al.,* 2012).

In this study, fish fed 0.1% Celmanax-containing diet, showed significantly higher serum lysozyme activity and plasma total antibody level. Previous studies showed that serum lysozyme activity could be used as an important biomarker to measure the innate immune response in fish. It has been reported that Lysozyme as a hydrolytic enzyme prevents bacterial growth by attacking peptidoglycan in bacterial cell walls (Galindo *et al*., 2003). Plasma total antibody level on the other hand is reflecting the plasma B-cells capability in recognizing and neutralizing the foreign bodies (Solem and Stenvik, 2006). Considering the outcome of both immunological biomarkers in this study, it may be suggested that a diet containing 0.1% Celmanax® might be a useful approach to enhance innate immunity and potentiate the humoral immunity against pathogenic microorganisms. In agreement with our findings recently Gunathilaka *et al.* (2015), reported that supplementation of diet with propolis resulted in remarkable lysozyme and myeloperoxidase activities and total immunoglobulin level (Gunathilaka *et al*., 2015).

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Numerous studies have demonstrated that both high temperature and hypoxia could be detrimental stressors in the fish industry and this could be especially important in coldwater fish species. The results in this study showed that all tested items of survival rate, plasma cortisol and glucose levels, which altered in hypoxic and/or high-temperature conditions, were regulated by the diet containing Celmanax® and the best results obtained when 0.1% concentration was provided. Although fishes in the experimental groups were not exposed simultaneously to both environmental stressors (Hypoxia and High temperature), it seems either stress factor elevate the plasma cortisol level and consequently plasma level of glucose. Many decades of investigation, however, suggest that these two stressors are acting synergistically and proposing an idea of'ecological surprises' in which both stressors individually and in much extend in combination form are leading to metabolic depression (McBryan *et al*., 2013; Olsvik *et al*., 2013). To explain how Celmanax® supplementation could successfully attenuate the hypoxia- and/or high temperature-increased cortisol and glucose level, it would be noteworthy to consider the fact that based on previous studies which were conducted on mammalians, it has been highlighted that during hypoxia and also high temperature, the microbiota alterations are inevitable (Atanu-Ghosh *et al*., 2014). Therefore, those alterations result in intestinal dysfunction, which prebiotics and in our study Celmanax® most likely could prevent from harmful microbiota changes and its consequently occurring events including survival rate, cortisol and glucose levels changes.

The last part of this study devoted to clarifying any potential and at the same time beneficial effect of Celmanax® on experimentally-induced Yersiniosis. The results revealed that during the two weeks of the Yersinia challenge, the rate of survival in the group of fish that fed a diet containing 0.1% Celmanax®, exceed 70% in comparison to the control group. The most reasonable explanation for such a remarkable effect may be related to the capability of given prebiotic in the prevention of pathogen proliferation and even toxin production (reduction of inflammatory processes in the liver and plasma cholesterol). In addition to the commonly described effects of prebiotics in stimulating the growth and activity of beneficial bacteria in the GUT- which we also witnessed in our study- there are other pathways in which prebiotics may have a positive role in the health issue including regulation of hepatic lipogenic enzymes by elevation of short-chain fatty acids production, modulation of mucin production, increase of lymphocytes in GUT-associated lymphoid tissues and peripheral blood and ultimately increase of antibody secretion (Markowiak and Slizewska, 2017). The results fully are in agreement with the known mechanism of action of prebiotics as the GUT microflora increase, serum immunological items provoking and ultimately significant increases in survival rate in Yersinia challenge improve the usefulness of Celmanax®. Other pathways which may Celmanax® supplementation

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resulted in prevention from Yersinia-induced death, could be its capacity in the enhancement of antibody production and in particular IgA production which in turn stimulate the phagocytic function of macrophages (Schley and Field, 2002). In conclusion; these data partly confirm the beneficial effects of Celmanax in rainbow trout. Moreover, the observed results indicated its mechanism of action, which attribute to its GUT microflora enrichment, serum lysozyme activity enhancement and total antibody level elevation. Increasing immunity and resistance to environmental alterations including oxygen and temperature changes along with remarkable potency in encountering bacterial challenge could be counted as beneficial effects of Celmanax in rainbow trout.

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Conflicts of interest

Authors have no conflict of interest on this work.

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