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Effects of essential oils of *Satureja bachtiarica* and *Nigella sativa* on the efficacy of lactococcosis vaccine in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Lactococcosis has been defined as acute septicaemia, which causes economic losses in farmed fish, especially in rainbow trout. This study was done to evaluate the effects of the essential oils of *Satureja bachtiarica* and *Nigella sativa* on the efficacy of lactococcosis vaccine in rainbow trout. A total number of 270 fishes with a mean weight of 120 g were obtained; they were randomly divided into nine groups, each with three replicates, after two weeks of adaptation. The groups were: no injection group, vaccine only group, DMSO injection group, vaccine with 50, 100, and 200 micrograms Intraperitoneal injection (IP) injection. Two, four, and six weeks after vaccination, serological and haematological parameters were evaluated. In the sixth week, 1.7×10^7 cfu as LD_{50} 96 hrs of *Lactococcus garvieae* were IP injected and the relative survival percentage was calculated. The results indicated that *N. sativa* essence is effective on the leukocyte population as the highest number of leukocytes were found in fish receiving high concentration of *N. sativa*. The relative survival rate of the studied fish decreased with decreasing concentrations of the *N. sativa* essential oil concentration, with a significant difference with control groups (p<0.05). However, using *S. bachtiarica* was not significantly effective on the relative survival rate of fish. The results of this study indicated that *N. sativa* essential oil can be used as adjuvant for *L. garvieae* vaccine, since it resulted in increasing leukocytes and the relative survival rate although *S. bachtiarica* was not effective on immune parameters of the studied fish.

Keyword: *Nigella sativa*, *Satureja bachtiarica*, Streptococcosis, Lactococcosis, Adjuvant.

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Introduction

Lactococcosis is an acute septicaemia affecting different fish species worldwide. *Lactococcus garvieae*, which is Gram-positive, non-motile, and non-spore forming ovoid coccus with α-hemolytic activity, is the causative agent of lactococcosis in economically important fish species (Eldar *et al.,* 1996; Vendrell *et al*., 2006). It has been also isolated from other animals including cows, dogs, cats, and buffalos (Collins *et al*., 1989; Carvalho *et al*., 1997). *L. garvieae* is also known as a zoonotic pathogen causing endocarditis in immunosuppressed persons, children, and elderly people (James *et al*., 2000; Mofredj *et al.,* 2000; Vinh *et al*., 2006).

Occurrence of *L. garvieae* in cultured rainbow trout has been reported in many countries including Australia, South Africa, Japan, Taiwan, England, Turkey, and Iran (Ghittino and Prearo, 1992; Palacios *et al*., 1993; Chen *et al*., 2001; Chang *et al*., 2002; Chem *et al*., 2002; Diller *et al*., 2002; Soltani *et al*., 2008). In Iran, lactococcosis was first reported by Akhlaghi and Keshavarzi (2003) in Fars Province. Since then, several epizootic outbreaks of lactococcosis have been reported in different areas of Iran (Soltani *et al*., 2008, Soltani and Tarahomi, 2009). This disease is now responsible for high mortalities and economic losses in rainbow trout farms.

Adjuvants are natural or synthetic compounds that are used in vaccination to enhance the immune response against the targeted pathogen. The function of adjuvants is to improve the presentation of antigen to immunocompetent cells (Dugenci *et al*., 2003).

There are a few previous studies on using adjuvants upon vaccination in fish. However, no study was found on using plants' essential oils as adjuvants in vaccination of fish.

Ravelo *et al*. (2006) studied the effects of different chemical adjuvants on formalin-killed aqueous *L. garvieae* vaccine. The relative percentage survival ranged from 45.7% to 86.9%. Their results indicated that vaccination with the Aquamun adjuvant seems to be the best method for protection of rainbow trout against lactococcosis. In another study, the effects of different adjuvant were studied on the efficacy of Salmonid Alpha Virus vaccine. The results showed that CpG/polyI:C could elevate immune responses (Thim *et al*., 2014).

Immunomodulatory effects of β-1, 3 glucan, levamisole, and Vitamins C and E in *Labeo rohita* were studied upon vaccination against *Edwardsiella tarda* by Sahoo and Mukherjee (2002). Their results revealed that using adjuvants increased the resistance to *E. tarda* and reduced the mortality rate.

In recent years, there has been increased interest in the use of medicinal plants and their derivatives in the prevention and treatment of fish diseases. These compounds increase resistance to pathogens by stimulating immune responses (Dugenci *et al.,* 2003). Various medicinal plants and their derivatives are known as immunostimulants. *Nigella sativa* and

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Satureja bachtiarica are known to have immunostimulating effects in fish (Kanter *et al.,* 2003; John *et al.,* 2007; Diab *et al.,* 2008). Therefore, this study is aimed to evaluate the effects of essential oils of *S. bachtiarica* and *N. sativa* on the efficacy of streptococcosis/lactococcosis vaccine in rainbow trout.

Material and methods

Fish samples

A total of 270 rainbow trout fish with an average weight of 120±6.7 g were obtained from a fish farm in Koohrang area, Chaharmahal va Bakhtiari Province. They were transported alive in bags containing 1/3 water and 2/3 oxygen to the Research Farm of Islamic Azad University of Shahrekord. The fishes were acclimatized in aerated ponds for 14 days with the following conditions: water temperature: 17.09±1.5°C, pH: 8.9±0.51, DO: 5.3±0.21 mg L^{-1}, ammonia: 0.020±0.005 mg L^{-1}, nitrite: 0.028±0.006 mg L^{-1}, TDS: 188.3±29.05 mg L^{-1}). They were then allocated into nine groups, each containing 30 fishes in three replicates. During the experiment, the fishes were fed with Kimiagaran Taghzieh extruded food three times per day. Essential oils of *N. sativa* and *S. bachtiarica* were obtained from medicinal plants and the Ethnoveterinary Research Centre, IAU, Shahrekord.

Experiment conditions

In Groups 1-6, the volume of 0.1 mL of the essential oils (50, 100 and 200 µg) dissolved in DMSO (Lot D-8418, Sigma Aldrich, USA) were Intraperitoneal injection (IP) injected as adjuvant with 0.1 mL of the streptococcus/lactococcus vaccine (ACECR[1], Iran). In Groups 7 and 8, vaccine and DMSO were singly injected and no injection was done in Group 9.

At the time of two, four, and six weeks after injection, blood samples were collected individually via caudal vein puncture. Fishes were anesthetized before sampling with clove oil at the concentration of 150 mg L^{-1}.

Each blood sample was divided into two halves, one in sterile heparin containing vial for haematological analysis and another without anticoagulant was centrifuged at 3,000 rpm for 5 min to collect serum (Dati *et al.,* 1996).

The antibody titre of the serum samples was determined using the micro agglutination method, as described by Roberson (1990). Wells with a button with fuzzy edges at the bottom were considered as positive, and wells with a sharp round precipitation were considered as negative.

Determination of total red blood cells (RBC×10^6 µL) and white blood cells (WBC×10^4 µL) was done using a haemocytometer after dilution with the Hayem solution. Hb was determined in units of grams per dl using the Hb detection kit (Pars Azmoon, Iran) using the spectrophotometer (Shimadzu,

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Japan) at 540 nm and in accordance with the cyamethaemoglobin method (Feldman *et al.*, 2000). PCV (%) was determined by centrifuging heparinized blood in capillary tubes at 7,000 g for 10 min in accordance with the method by Blaxhall and Daisley (1973).

For differential count of leukocytes, blood smear slides were prepared and stained with Wright-Giemsa. The slides were observed by using an oil emersion lens under a light microscope. At least 100 leukocytes were counted, and the percentage of each cell was calculated (Hoseinifar *et al.*, 2010).

Red blood cell indices, such as mean cell haemoglobin (MCH, pg), mean cell haemoglobin concentration (MCHC, %), and mean corpuscular volume (MCV, fL), were calculated according to Houston (1990).

The efficacy of vaccine, with or without adjuvants, was determined by IP injections of vaccinated and unvaccinated fish with *Lactococcus garvieae* in six weeks' post-vaccination period. A volume of 0.1 mL was applied to each fish equal to 1.7×10^7 cfu as LD_{50} 96 hrs of *L. garvieae*, which was determined before the experiment. The mortality of fish was recorded up to 10 days after inoculation. The relative percentage survival (RPS) was calculated using the following equation: RPS= [1-(percent of mortality in vaccinated fish/ percent of mortality in unvaccinated fish) ×100]. This was in accordance with Ellis (1988).

Statistical analysis

The data was compared by using Duncan test through SPSS version 20.0 statistical software (SPSS Inc., USA). The differences were considered statistically significant at $p\leq 0.05$.

Results

Two weeks after vaccination, leukocytes population in the receiving essential oil group increased compared to the control group. So, the maximum rate of white blood cells was observed in the 200 mg of *N. sativa* group. Leukocytes in the fish receiving 200 and 50 mg of *N. sativa* were significantly different compared to other groups ($p<0.05$). Highest Neutrophil population was observed in *N. sativa*, showing significant difference with other groups ($p<0.05$). However, there was no significant difference in the mean values of lymphocyte number. The results of the second week also revealed that no significant difference was observed between the mean values of haematocrit, the number of red blood cells, and haemoglobin of the studied fish (Table 1).

In the fourth week, leukocytes population increased compared to the second week after vaccination. The mean leukocytes in essential oil groups were higher than the control fish with statistical difference ($p<0.05$) in the 200 and 50 mg *N. sativa* groups.

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Table 1: Hematological parameters of the vaccinated and non-vaccinated fish after 2 weeks.

Antibody level	MCHC (gr/dl)	MCH (pg)	MCV (Fl)	Hb (gr/dl)	RBC x 10^6	Hct (%)	Basophil (%)	Eosinophil (%)	Monocyte (%)	Lymphocyte (%)	Neutrophil (%)	Lucocytes / microliter	Group	
10^b	28.9± 0.5^a	32.3± 0.3^a	111.5 ± 1.8^a	13.9± 1.6^a	4.3± 0.5^a	47.8± 4.9^a	0.6± 0.7^a	1.6± 1^{ab}	4.2± 1.1^{ab}	75.8± 2.3^a	17.6± 1^a	14581.2± 1486.8^b	A	*S. bachtiarica*
10^b	29.3± 0.4^a	32.1± 0.3^a	109.5± 2.6^a	15.1± 1.7^a	4.7± 0.5^a	51.5± 5.2^a	1± 0.7^a	1.6± 0.5^{ab}	3± 0.7^a	76.6± 2.1^a	17.75 ± 1.8^a	13100± 1502.1^{ab}	B	
10^b	27.5± 2.5^a	32± 1.1^a	116.8± 8.5^a	12.8± 3.2^a	3.9± 1^a	42.8± 10^a	0.75± 0.4^a	1.6± 0.5^{ab}	3± 0.7^a	76.6± 2.1^a	17.3± 2^a	14306.2± 2900.4^b	C	
10^b	28.8± 0.4^a	32.6± 0.4^a	113± 3.1^a	13± 1.4^a	4± 0.4^a	45.2± 4.3^a	1.1± 0.8^a	1.8± 0.6^{ab}	3.8± 1.1^{ab}	74.8± 3.5^a	18.3± 2.6^b	15956± 3128.3^c	A	*N. sativa*
10^b	29.1± 0.6^a	32.3± 0.5^a	111.1± 4.5^a	14.3± 2.6^a	4.4± 0.8^a	49± 8^a	0.6± 0.7^a	1.5± 0.9^{ab}	4.1± 1.1^{ab}	76.8± 2.8^a	16.8± 1.2^a	13688± 1696.6^{ab}	B	
10^b	29± 0.9^a	32.5± 0.8^a	112.5± 6.9^a	13.9± 2.5^a	4.2± 0.8^a	47.7± 7.7^a	0.7± 0.7^a	1.8 ± 0.8^{ab}	5.3± 1.6^b	73.5± 2^a	18.5± 2.5^b	15488± 2940.2^c	C	
10^b	29.3± 0.1^a	32± 0.3^a	109.2± 1.7^a	14.8± 0.7^a	4.6± 0.2^a	50.5± 2.3^a	0.5 ±0.5^a	2 ± 0.8^{ab}	4.5± 2^{ab}	75.8± 4.5^a	17.2± 1.7^a	14463± 1641.8^b	D	
0^a	29.2± 0.5^a	32.2± 0.2^a	110.1± 2.7^a	14.8± 2.1^a	4.6± 0.7^a	50.5± 6.5^a	1.2± 0.9^a	1.2 ± 0.9^a	4.2± 2^{ab}	77± 4.3^a	16.2± 1.5^a	12875± 499.1^a	E	No essential oils
0^a	28.6± 0.9^a	32.4± 0.5^a	113.5± 5.5^a	12.7± 2.2^a	3.9± 0.7^a	45.5± 6.8^a	0.8± 0.7^a	2.5 ± 0.7^b	4.2± 0.4^{ab}	75.4± 0.4^a	17.5± 1.2^a	13263± 1446.3^{ab}	F	

A: Vaccine with 200 µg essential oil, B: Vaccine with 100 µg essential oil, C: Vaccine with 50 µg essential oil, D: Vaccine with no essential oil, E: DMSO, F: No injection.

Differential counts of white blood cells showed no significant difference in the mean cell count, except for neutrophils, in the group of 50 *S. bachtiarica* (p<0.05) (Table 2).

In the sixth week after vaccination, the same as the second and fourth weeks, the maximum rate of leukocytes was observed in the *N. sativa* group and in concentration of 200 mg with significant difference compared to the other groups (p<0.05). Based on the results, in the sixth week, the mean percentages of neutrophil, lymphocyte, eosinophil and basophil had no significant differences in different groups, but there was a significant increase in haematocrit, red blood cells, and haemoglobin, with increased concentration of essential oils (p<0.05). The same as the second and fourth weeks, the antibody level of non-vaccinated fish and the fish receiving only DMSO was equal to zero, showing significant differences (p<0.05) compared to that in the essential oil groups.

After the inoculation of the bacteria, non-vaccinated fish died within seven days, but other groups had fewer losses and higher survival time. The maximum RPS rate was observed with the concentration of 200 mg *N. sativa*. The RPS rate for Groups A-F was 73.3, 62.5, 62.5, 67, 67, and 62.5, respectively. It was 62.5, 0, and 0 for Groups G, H, and I, respectively. There was significant difference between groups A and control groups (p<0.05) (Charts 1 and 2).

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Table 2: Hematological parameters of the vaccinated and non-vaccinated fish after 4 weeks.

Antibody level	MCHC (gr/dl)	MCH (pg)	MCV (Fl)	Hb (gr/dl)	RBC x 10^6	Hct (%)	Basophil (%)	Eosinophil (%)	Monocyte (%)	Lymphocyte (%)	Neutrophil (%)	Lucocytes / microliter	Group	
40^{c}	29± 0.5 ^{a}	32.1± 0.2^{a}	110.7± 2.8^{a}	13.8± 1.9^{b}	4.3± 0.6^{abc}	47.6± 5.9^{abc}	0.5± 0.5 ^{a}	1.3± 0.5 ^{a}	4.1± 1.4 ^{a}	76.8± 3.2 ^{a}	17.1± 1.4^{a}	16450± 1983.4^{ab}	A	*S. bachtiarica*
20^{b}	29.3± 0.6 ^{a}	32.2± 0.4 ^{a}	110.1± 4^{a}	15± 2.2^{c}	4.6± 0.7^{c}	51± 6.8^{c}	0.5± 0.5 ^{a}	1.3± 0.5 ^{a}	4± 1.2 ^{a}	76.3± 3.4 ^{a}	17.8± 1.9 ^{a}	16113.3± 3782.1^{ab}	B	
20^{b}	29.5± 1.2 ^{a}	32.3± 0.2 ^{a}	110.8± 2.2^{a}	14.2± 1.5^{bc}	4.4± 0.5^{bc}	48.6± 4.7^{bc}	0.6± 0.5 ^{a}	1.5± 0.5 ^{a}	4.6± 1 ^{a}	73.3± 3.9 ^{a}	19.8± 3.4 ^{b}	17933.3± 2724.6^{ab}	C	
20^{b}	29.3± 0.6 ^{a}	32.3± 0.6 ^{a}	110.4± 4.4^{a}	14.8± 2.4^{c}	4.6± 0.8^{c}	50.5± 7.3^{b}	0.8± 0.7 ^{ab}	1.5± 0.5 ^{a}	4.5± 1.5 ^{a}	74.8± 4.5 ^{a}	18.3± 2.9 ^{a}	18808± 2743.6^{b}	A	
20^{b}	28.2± 0.7 ^{a}	32.7± 0.4 ^{a}	115.8± 4.6^{a}	11.5± 1.9^{a}	3.5± 0.6^{a}	40.5± 5.7^{a}	0.3± 0.5 ^{a}	1.6± 0.8 ^{a}	4.5± 1.3 ^{a}	75± 3.8 ^{a}	18.5± 2.4 ^{a}	17642± 3807.7^{ab}	B	*N. sativa*
20^{b}	29.5± 0.2 ^{a}	31.9± 0.2 ^{a}	108.6± 1.4^{a}	15.5± 1^{c}	4.8± 0.3^{c}	52.6± 3^{c}	1.1± 0.4 ^{ab}	1.6± 0.5 ^{a}	5.6± 0.8 ^{a}	73 ±2.1 ^{a}	18.3± 1.7 ^{a}	18967± 2098.5^{b}	C	
20^{b}	29.2± 0.5 ^{a}	32± 0.2 ^{a}	109.4± 2.7^{a}	14.9± 1.9^{c}	4.6± 0.6^{c}	50.7± 5.9^{b}	0.5± 0.5 ^{a}	1.5± 0.5 ^{a}	4± 1.8 ^{a}	76.3± 2.8 ^{a}	17.75± 0.9 ^{a}	14250± 1049.6^{a}	D	
0^{a}	28.5± 0.6 ^{a}	32.7± 0.4 ^{a}	114.5± 4.1^{a}	12± 1.6^{ab}	3.6± 0.5^{ab}	42± 5^{ab}	1± 0.8^{ab}	2± 0.8 ^{a}	4.7± 1.7 ^{a}	74.3± 5.1 ^{a}	18 ± 2.1 ^{a}	14313± 2805.8^{a}	E	No essential oils
0^{a}	28.8± 0.4 ^{a}	32.7± 0.4 ^{a}	113.3± 3.2^{a}	13± 1.5^{abc}	4± 0.5^{abc}	45.2± 4.7^{abc}	1.5± 0.5^{b}	1.7± 0.9 ^{a}	4.5± 1.2 ^{a}	75.3± 1.2 ^{a}	17 ± 1.4 ^{a}	13300± 1553.5^{a}	F	

A: Vaccine with 200 µg essential oil, B: Vaccine with 100 µg essential oil, C: Vaccine with 50 µg essential oil, D: Vaccine with no essential oil, E: DMSO, F: No injection.

Table 3: Hematological parameters of the vaccinated and non-vaccinated fish after 6 weeks.

Antibody level	MCHC (gr/dl)	MCH (pg)	MCV (Fl)	Hb (gr/dl)	RBC x 10^6	Hct (%)	Basophil (%)	Eosinophil (%)	Monocyte (%)	Lymphocyte (%)	Neutrophil (%)	Lucocytes / microliter	Group	
40^{b}	29.6± 0.3 ^{a}	32± 0.4 ^{a}	108± 2.4 ^{a}	16.1± 1.5 ^{c}	5± 0.5 ^{c}	54.5± 4.7^{c}	0.5± 0.5^{ab}	1.25± 0.5 ^{a}	5.5± 3 ^{a}	75.5± 4.6 ^{a}	18± 2.7^{a}	13975± 933.7^{b}	A	*S. bachtiarica*
40^{b}	28.6± 0.4 ^{a}	32.5± 0.4 ^{a}	112.2± 3.9 ^{a}	13.7± 1.8 ^{b}	4.2± 0.6^{ab}	47.25± 5.3^{abc}	0.5± 0.5 ^{ab}	2.2± 0.5 ^{a}	4.2± 1.5 ^{a}	75.5± 4.9^{a}	17.5± 3.4^{a}	13575± 1998.5^{b}	B	
40^{b}	28.9± 0.6 ^{a}	32.3± 0.6 ^{a}	111.8± 4.6 ^{a}	13.6± 2.5^{b}	4.2± 0.8^{ab}	47± 7.6 ^{abc}	0.75± 0.5^{ab}	1.5± 1 ^{a}	5.2± 1.2 ^{a}	74.2± 2.2^{a}	18.2± 2^{a}	13675± 1575 ^{b}	C	
40^{b}	29.9± 0.7 ^{a}	31.7± 0.6 ^{a}	106.3± 4.2 ^{a}	15.6± 1^{bc}	4.9± 0.4^{bc}	52.2± 3^{bc}	0.2± 0.5 ^{a}	1±0 ^{a}	6.5± 1.7 ^{a}	74.5± 1^{a}	17.7± 0.9^{a}	16475± 1857.2^{c}	A	*N. sativa*
40^{b}	32.4± 4.8 ^{a}	33.1± 0.5 ^{a}	103.7± 13.9 ^{a}	13.9± 2.1^{b}	4.2± 0.6^{ab}	43± 3.6 ^{ab}	0.5± 0.5 ^{ab}	0.7± 0.9 ^{a}	5.2± 0.5 ^{a}	76.3± 2.2^{a}	17.7± 0.5^{a}	13475± 943.8 ^{b}	B	
40^{b}	29.2± 0.4 ^{a}	32.4± 0.3 ^{a}	110.7± 2.1 ^{a}	14.5± 1.6^{bc}	4.4± 0.5^{b}	49.5± 4.9^{b}	0.5± 0.5 ^{ab}	1.5± 0.5 ^{a}	3.7± 1.7 ^{a}	77± 1.4^{a}	17.2± 2^{a}	13350± 1292.9 ^{b}	C	
40^{b}	27.9± 0.3 ^{a}	33.3± 0.2 ^{a}	119± 1.3 ^{a}	10.5± 0.7 ^{a}	3.1± 0.2 ^{a}	37.5± 2.1 ^{a}	0.5± 0.5 ^{ab}	1±0 ^{a}	4±0 ^{a}	76.5± 2.1^{a}	18.5± 2.1^{a}	13425± 671.7 ^{b}	D	
0^{a}	29.4 ^{a}	31.9 ^{a}	108.5 ^{a}	15± 1.1 ^{bc}	4.7± 0.2^{bc}	51± 2.3^{bc}	1.5± 0.7^{b}	2±0 ^{a}	4.5± 0.7 ^{a}	76± 1.4^{a}	16± 1.4^{a}	11650± 1060.7 ^{a}	E	No essential oils
0^{a}	28.5± 1.2 ^{a}	32.6± 1 ^{a}	114.5± 8.5 ^{a}	12.3± 3.7 ^{ab}	3.8± 1.2 ^{ab}	43± 11.3 ^{ab}	0.5± 0.7 ^{ab}	1±0 ^{a}	5± 2.8 ^{a}	74± 1.4^{a}	19.5± 2.1^{a}	11450± 1104.2 ^{a}	F	

A: Vaccine with 200 µg essential oil, B: Vaccine with 100 µg essential oil, C: Vaccine with 50 µg essential oil, D: Vaccine with no essential oil, E: DMSO, F: No injection.

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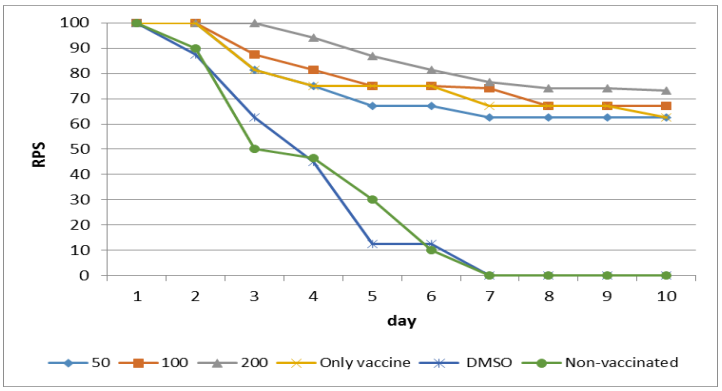


Chart 1: RPS in fish received *Nigella sativa* comparing with control groups.

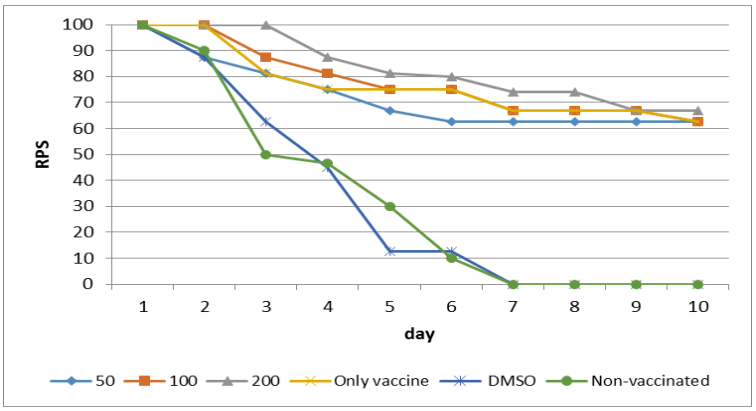


Chart 2: RPS in fish receiving *Satureja bachtiarica* compared with control groups.

Discussion

Lactococcosis is considered as an important bacterial septicaemia of fishes in Iran and the world. *L. garvieae*, is gram-positive rod shape bacteria, which is more virulent at temperature more than 15°C, the high-density of fish, low oxygen, and high organic material of water. In recent years, this disease has been reported from different parts of Iran (Akhlaghi and Keshavarzi, 2003; Soltani *et al*., 2008; Raissy and Moumeni, 2016 and Raissy *et al*., 2016). Lactococcosis is now considered as the most important bacterial disease of rainbow trout in Iran with great economic losses (Raissy *et al*., 2016).

This is the only fish diseases the vaccine for which has been made in Iran. In recent years, lactococcosis vaccine was prepared using native strains in the University of Tehran, which could be usable as immersion and IP injection.

Medicinal plants have known and proven effects on the immunity systems of different animals (Bensky and Gample, 1993). These effects are affected due to non-specific immune stimulation either cellular or humoral immune of the host. So far, many studies have been done on

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immunostimulants in fish. Among them, medicinal plants have been recommended due to proper effect and lack of side effects repeatedly (Dugenci *et al*., 2003; Ghasemi Pirbaloti *et al*., 2011; Alishahi *et al.*, 2012). This matter regarding indiscriminate and uncontrolled use of antibiotics and disinfectants is very important.

In the present study, essential oils of *S. bachtiarica* and *N. sativa* were used as adjuvant with lactococcosis vaccine and the results were assessed in the second, fourth, and sixth weeks after vaccination. The results in the second and fourth weeks showed that the highest number of white blood cells was seen in fish that received 200 and then 50 mg of *N. sativa*. In the sixth week, the maximum count again was seen in the 200 mg *N. sativa* group. These findings suggest that the effects of *N. sativa* on the number of white blood cells agree with results obtained in other studies (Dugenci *et al*., 2003; Khondoker *et al*., 2016). Altinterim and Dorucu (2013) reported enhancement of the immune system of trout fish after the use of *N. sativa*. Their findings are inconsistent with the results of Alishahi and Mesbah (2012) on the immunogenicity of *N. sativa* in Goldfish.

The results of this study showed that the use of essential oils has increased the neutrophils population, especially in the first two weeks. In a similar study, Ghasemi Pirbalouti *et al*. (2011) showed that the use of essential oils of *S. bachtiarica* and *S. khuzestanica*, *Thymus vulgaris*, *Mentha longifolia* and *Dracocephalum multicaule* led to a significant increase in the population of white blood cells, especially neutrophils in rainbow trout. This study shows a decrease in lymphocytes and monocytes population, despite the relative increase in the general population of white blood cells. The reason for this issue is an increase in the percentage of neutrophils in the blood, which naturally is associated with a decline in the population of cells. This finding is fully consistent with the findings of Ghasemi Pirbalouti *et al*. (2011).

The highest rate of RPS was found in the 200 mg *N. sativa* group, showing a significant difference with that in the control group ($p<0.05$). There was no significant difference in the RPS of the vaccinated fish with or without the adjuvant except 200 mg *N. sativa*. This is supported by the high level of antibody in fish receiving 200 mg *N. sativa* with vaccine.

In a similar study, Khatun *et al*. (2015) reported the minimum mortality rate in fish that received *N. sativa* compared to other essential oils which is consistent with the results of this study.

Overall, the results suggest that essential oil of *N. sativa* is effective in promoting the quality of streptococcosis/lactococcosis vaccine of trout fish, so it can be used as an adjuvant. Use of high concentrations of *N. sativa* led to an increase in the white blood cell population and the rate of RPS although there was no significant increase in the above values in fishes receiving essential oil of *S. bachtiarica*.

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Therefore, unlike *N. sativa*, *S. bachtiarica* is not effective in promoting the immunity caused by vaccine.

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