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Differential MHC-П, IL-8 and IgM genes expression in the liver, head Kidney and gill tissues of juvenile rainbow trout (Oncorhynchus mykiss) immunized by inactive trophonts of Ichthyophthirius multifiliis

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► ***Original article***

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ABSTRACT

Background: The current study was designed to compare the genes expression levels of *MHC-П*, *IL-8* and *IgM* in the head kidney and liver (central organs), as well as gill tissue (mucosal surface) of juvenile rainbow trout. ***Materials and Methods:*** Juvenile rainbow trout were immunized with gamma-irradiated and formalin inactive trophonts with/without alginate nanoparticles against *Ichthyophthirius multifiliis*. On day 20 after bath-immunization, fish in all the treatments, except negative control, were exposed to live trophonts. On the 30th day, fish tissues were analyzed by real-time PCR. ***Results:*** The *IL-8* expression up-regulated in the kidney, hepatic and gill of immunized fish with irradiated and formalin inactive trophonts plus alginate nanoparticles and non-immunized fish challenged with live trophonts (*P<0.05*). The *MHC-II* showed up-regulation in gill and kidney of immunized fish and non-immunized fish challenged with live trophonts while hepatic *MHC-П* was up-regulated in immunized fish with formalin inactive trophonts plus alginate nanoparticles (*P<0.05*). A significant increase in the *IgM* expression was detected in gills of immunized fish with irradiated trophonts with/without alginate nanoparticles and formalin inactive trophonts plus alginate nanoparticles (*P<0.05*). The renal *IgM* showed up-regulation in all of treatments (*P<0.05*). A significant increase in the hepatic *IgM* expression was in immunized fish via irradiated trophonts with/ without alginate nanoparticles (*P<0.05*). ***Conclusion:*** These findings indicated that the higher expression of *IgM, MHC-II and IL-8* at the gill, kidney and liver could prove the potential of the entire treatments to enhance both mucosal and systemic immune responses in immunized fish with gamma irradiated trophonts against *I. multifiliis.*

Keywords: *Rainbow trout, gamma- irradiated Ichthyophthirius multifiliis, alginate nanoparticles, systemic immune response.*

INTRODUCTION

The ciliated protozoan *Ichthyophthirius multifiliis* (*I. multifiliis*) is a large and rather important parasite of freshwater fish that causes 'white spot disease' which is leading to sudden death of fish worldwide [1, 2]. Chemotherapy by chemical agents is difficult to administer and useless after its penetration of skin and gills. Prior studies demonstrated that fish develop both systemic and mucosal immune responses and protection after treatment with *I. multifiliis* antigens [3, 4, 5, 6]. Thus, immunization against *I. multifiliis* and immunotherapies are good alternative methods for chemotherapy [2]. Besides, applying a convenient adjuvant can increase the potential of vaccines. The biodegradable and biocompatible polymers,

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such as alginate, have promising and safety systems to be used as adjuvant. This polysaccharide is found naturally in brown algae and some bacteria, such as *Azotobacter vinelandii* and *Pseudomonas* [7]. The adjuvant effects of alginate were proved in previous studies [8 - 14].

Although, *I. multifiliis is parasitic on the mucosal surfaces of the skin, gills* and nose, innate adaptive immune responses can also be induced in systemic organs of some fish species following this sub-lethal infection [15]. In our previous study, an up-regulation of *IgM* in the skin of the rainbow trout from day 20 to day 30 after immunization with gamma-irradiated and formalin inactive trophonts plus alginate nanoparticles were observed [16]. Since there is no information about the immunoprotective role of these inactive trophonts on systemic immune responses, present study was designed. In this study, we investigated the expression genes of renal, hepatic (as central organs) and gill (as mucosal surface) *MHC-Π*, *IL-8* and *IgM* in the context of treated rainbow trout with a gamma irradiated *I. multifiliis* trophonts plus alginate nanoparticles compared to formalin inactive *I. multifiliis* and live trophonts to evaluate the mucosal and systemic immunity against *I. multifiliis*.

MATERIALS AND METHODS

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*) (mean body weight 74 - 40 g) were kept in 300 L running water (flow rate 0.5 Lit/s) polyethylene tanks. It was continuously supplied with aerated water, temperature 15 ± 1 ºC, dissolved oxygen 5.2 ppm under natural photoperiod (10L:14D). Fish were acclimatized to the experimental rearing condition for 14 days on a commercial pelleted diet (Behparvar, Iran).

Gamma-irradiated and formalin inactive trophonts procedure

Gamma-irradiated trophonts were prepared as described previously [17]. Trophonts of *I. multifiliis* used in this study were obtained from an infected pet fish. In brief, fifty fish were infected with *I. multifiliis* via a high dose of collected live trophonts by immersion method. Exposure was performed in the dark for 8 hrs. Fish were kept for 5 days at 20 °C and then trophonts were collected with 200-mesh sieve, examined under light microscope for survival and counted. Live trophonts were immediately used for preparing the gamma-irradiated or formalin inactive trophonts. For irradiation, gamma cell "Nordian", model 220 with dose rate of 0.22 Gy/sec and 20469 Ci activities and a dose of 170 Gy was used. For formalin inactivation, live trophonts were suspended in 3% formalin, incubated for 2 hrs at room temperature, and centrifuged at 3000 × g for 2 min. Then, the supernatants were removed and washed 3 times with 1 ml of 0.15 M sterile phosphate buffered saline (PBS) (pH=7.4). After washing, the formalin-treated trophonts were harvested by centrifugation at 3500 × g for 3 min [16].

Alginate nanoparticles

Alginate nanoparticles were prepared on the basis of our previous report [18]. Ergosan, (Schering Plough Aquaculture, UK) was suspended in sterile phosphate buffered saline (0.15 M, pH=7.2), sonicated on ice for 30 min and centrifuged at 5000 × g, for 15 min. After precipitation in 2.5 volumes of 96% ethanol and heating at 40˚C, the dried precipitate was then milled to the mesh size of 53–125 µm. Remaining powder was irradiated by cobalt-60 gamma irradiator (PX-30 – IssIedovapel, Russia) at a dose 30 kGy [18, 19]. Dosimetry was performed with Fricke reference standard dosimetry system.

Fish treatment design

For the treatment procedures, fish were randomly allocated between 18 triplicate tanks at a density of 30 fish per 300 L aquarium and maintained continuously aerated free-flowing dechlorinated freshwater (at total 540 fish). The fish were treated for separate aquarium immersion (Dose rate = 100 gamma-irradiated/ formalin inactive trophonts per 150 gram of fish body weight). The gamma-irradiated/formalin

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inactive trophonts and nanoparticles were applied to the fish as follows:

Control (C): Healthy fish as a negative control was kept untreated until termination of the experiment.

Treatment 1 (T1): Non-immunized fish challenged with 1000 live trophonts (as a positive control).

Treatment 2 (T2): Fish immunized with gamma-irradiated (170 Gray) trophonts.

Treatment 3 (T3): Fish immunized with gamma-irradiated (170 Gray) trophonts plus alginate nanoparticles.

Treatment 4 (T4): Fish immunized with formalin (3%) inactive trophonts.

Treatment 5 (T5): Fish immunized with formalin (3%) inactive trophonts plus alginate nanoparticles.

All the treatments, except C and T1, were administered for the second time on day 10 after the first immunization.

Challenge trials

On day 20 after the first immunization, the water flow was turned off, water reduced to 10 L in each tank and fish in all the treatments, except control group (C), were exposed to 10000 live trophonts with aeration, via bath method. Post challenge mortalities were recorded daily in both treated and the control groups for 3 weeks. During 3 weeks of experiment, percentage of survival rate was calculated for all treatments according to the following formula:

Survival rate (%) = 100 (Live juvenile fish – Dead juvenile fish) / Live juvenile fish

Sampling

All samples for this study were taken the same method described by Sigh et al., (2004)[5]. Five fish were sampled from each group at the 30th day following the first immunization. They were gently transferred to a small plastic aquarium containing mild anesthetic (MS 222, 20 mg/L). In the laboratory, fish were killed quickly by overdose of MS 222 (300 mg/L) and different tissues, including head kidney, liver and gill were aseptically dissected and subsequently snap-frozen in liquid nitrogen.

RNA extraction and cDNA synthesis

RNA extraction from the tissue samples was performed using TRIzol Reagent (Sigma-Aldrich), according to the manufacturer's instructions. Two µL of total extracted RNA from each sample were used in cDNA synthesis. The cDNA was generated using 1 µL of random hexamer primer (2 µg/L), 10 µL of 2× first standard reaction, 10 mM $Mgcl_2$, 1mM dNTPs and 2 µL reverse transcriptase. The reactions were incubated at 25˚C for 10 min, follow by 50 min at 50˚C and finally, 85˚C for 5 min and stored at -20˚C until further use. To assess PCR efficiency, serial dilutions of standard cDNA preparation were used to generate the standard curve for each primer set.

Quantitative PCR

All qPCR assays were carried out in the Step One™ Real-Time PCR System (Applied Biosystems, USA). The reactions contained 10 µl SYBR® Premix Ex Taq™ (Tli RNase H Plus), ROX plus (TaKaRa, Japan), 1 µl of cDNA, 0.5 µl of forward and reverse primer (100 nM) and filled up with ultra-pure water to a final volume of 20 µl. All samples were run in triplicate. The quantitative PCR thermal cycling conditions were: initial incubation step at 50˚C for 2 min and at 95˚C for 2 min followed by 40 amplification cycles of 15 s at 95˚C and 1 min at 60˚C. In order to detect the presence of non-specific amplification, control reactions without template were included for each primer set. At the end of each cycle, DNA melting curve analysis was performed in order to confirm the specificity of the PCR products.

Gene expression of the samples compared to the control was calculated according to the following equation, using RESC009 QPCR software (Qiagen, USA), and the Pfaffl method [20].

$$\text{Ratio} = (\epsilon_{target})\Delta c_{T\ target}\ (\text{control-sample}) / (\epsilon_{RPS11})\ \Delta c_{T\ target}\ (\text{control-sample})$$

The PCR primers were designed using the primer 3 program based on sequences deposited in the Gene Bank (primer sequences and amplicon length listed in table 1). The primer

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sets included genes of immunoglobulin (IgM), cytokine (IL-8) and cell receptor (MHC-Π). B-actin was selected as a reference gene and subsequently for sample normalization [5]. All primers were purchased from sigma Aldrich.

Table 1. The sequences of the forward and reverse primer as well as the amplicon length

Gene	Sequence (5–3)	Size of amplicon (bp)	Accession number
IL8.F	GAATGTCAGCCAGCCTTGTC	162	AJ279069
IL8.R	TCCAGACAAATCTCCTGACCG		
IgM.F	TGCGTGTTTGAGAACAAAGC	107	AH014877.2
IgM.R	GACGGCTCGATGATCGTAAT		
MHC-II.F	ATGTCGATGCCAATTGCCTTCTA	236	U20943
MHC-II.R	TGTCTTGTCCAGTATGGCGCT		
β-Actin.F	CAGCCCTCCTTCCTCGGTAT	110	NM_001124235.1
β-Actin.R	AGCACCGTGTTGGCGTACA		

Statistical analysis

All values were given as mean ± SD, which were means with 95% confidence intervals in triplicate. The results were subjected to variance analysis (ANOVA) followed by least significant differences (Tukey) test. Correlation coefficients were significant with $P < 0.05$.

RESULTS

Figure 1 shows the results of percentage survival of juvenile rainbow trout for 3 weeks after the challenge trial. The results obtained revealed that the percentage of fish that survived in the all groups were T1 = 47.23 ± 5.12 %, T2 = 60.12 ± 4.13 %, T3 = 83.45 ± 4.51 %, T4 = 66.01 ± 5.31 %, T5 = 76.82 ± 4.62 % and 90.67 ± 4.82 % for the negative control (C) that was healthy fish. The result indicated that the highest mortality rate was seen in the positive control group (T1) while the lowest mortality rate (83.45 ± 4.51%) in immunized fish with gamma-irradiated trophonts plus alginate nanoparticles (T3). There was no significant difference of mortality rate ($P = 6.61$) between T3 and negative control group (C).

In this study, expression profiles of cytokine, *IL-8*, gene in the liver, gill and head kidney tissues of rainbow trout after 30 days following the first immunization are shown in table 2. The renal *IL-8* showed an up-regulation at immunized fish with irradiated trophonts (T2), gamma-irradiated trophonts plus alginate nanoparticles (T3) and formalin inactive trophonts with alginate nanoparticles (T5) but hepatic *IL-8* were significantly up-regulated at irradiated trophonts treatment (T2), gamma-irradiated trophonts plus alginate nanoparticles treatment (T3), formalin inactive trophonts plus alginate nanoparticles treatment (T5) and at rainbow trout infected with live trophonts of *I. multifiliis* (T5) (P<0.05). *IL-8* in gill changed significantly in all treatments to negative control group (C) (P<0.05) (table 2).

Renal and gill *MHC-Π* were significantly up-regulated at infected fish with live trophonts (T1) and immunized fish with gamma-irradiated trophonts (T2), gamma-irradiated trophonts plus alginate nanoparticles (T3) and formalin inactive trophonts plus alginate nanoparticles (T5) (P<0.05) (figures 2 and 3). The hepatic *MHC -Π* was down-regulated at gamma-irradiated trophonts treatment (T2), gamma-irradiated trophonts plus alginate nanoparticles treatment (T3) significantly compare to formalin inactive trophonts plus alginate nanoparticles treatment (T5) (P<0.05) (table 3).

The renal *IgM* showed expression with significant up-regulation at gamma-irradiated trophonts treatment (T2), gamma-irradiated trophonts plus alginate nanoparticles treatment

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(T3) formalin inactive trophonts treatment (T4) and formalin inactive trophonts plus alginate nanoparticles treatment (T5) and hepatic IgM was up-regulated at gamma- irradiated trophonts treatment (T2), irradiated trophonts plus alginate nanoparticles treatment (T3) formalin inactive trophonts plus alginate nanoparticles treatment (T5) (P<0.05) (table 4). The gene of *IgM* in gill exhibited a significantly increase in mRNA expression level at gamma-irradiated trophonts plus alginate nanoparticles treatment to the other groups (T3) (P<0.05) (table 4).

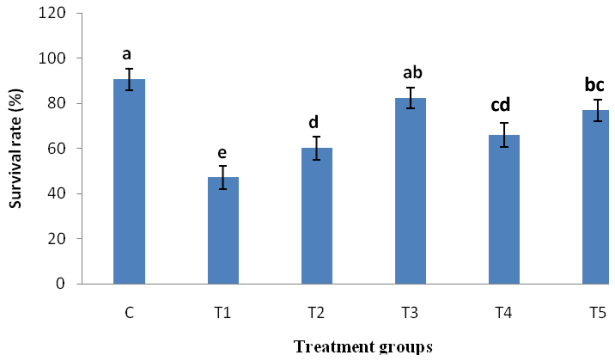


Figure 1. The survival rate (%) of juvenile rainbow trout in different treatments; C: Healthy fish, negative control; T1: Non -immunized rainbow trout challenged with live trophonts of Ichthyophthirius multifiliis; T2: Rainbow trout immunized via gamma-irradiated trophonts; T3: Rainbow trout immunized via gamma-irradiated trophonts plus alginate nanoparticles; T4: Rainbow trout immunized via formalin inactive trophonts; T5: Rainbow trout immunized via formalin inactive trophonts plus alginate nanoparticles.

Table 2. Expression of IL-8 gene in the liver, head kidney and gill of treated juvenile rainbow trout against Ichthyophthirius multifiliis relative to the β-actin gene during 30 days. Different lower case letters (a–c) within the same row (different tissues) indicate significant differences (P< 0.05).

Treatments / Tissues	C	T1	T2	T3	T4	T5
Head kidney	0.33± 0.13^{c}	0.61± 0.21^{abc}	1.26± 0.5^{a}	1.23± 0.33^{a}	0.65± 0.12^{b}	0.98± 0.14^{a}
Liver	0.35± 0.12^{b}	0.85± 0.33^{a}	1.11± 0.09^{a}	0.91± 0.36^{a}	0.49± 0.22^{ab}	0.78± 0.18^{a}
Gill	0.49± 0.05^{b}	1.42± 0.31^{a}	1.35± 0.42^{a}	1.01± 0.36^{a}	1.08± 0.33^{a}	1.09± 0.32^{a}

Values are represented as means ± SEM of three replicates treatments. C: Healthy fish, negative control; T1: Non-immunized rainbow trout challenged with live trophonts of Ichthyophthirius multifiliis; T2: Rainbow trout immunized via irradiated trophonts; T3: Rainbow trout immunized via irradiated trophonts plus alginate nanoparticles; T4: Rainbow trout immunized via formalin inactive trophonts; T5: Rainbow trout immunized via formalin inactive trophonts plus alginate nanoparticles.

Table 3. Expression of MHC-II gene in the liver, head kidney and gill of treated juvenile rainbow trout against Ichthyophthirius multifiliis relative to the β-actin gene during 30 days. Different lower case letters (a–c) within the same row (different tissues) indicate significant differences (P< 0.05).

Treatments / Tissues	C	T1	T2	T3	T4	T5
Head kidney	1.01± 0.32^{b}	1.62± 0.6^{ab}	1.45± 0.3^{ab}	2.03± 0.4^{a}	0.95± 0.31^{b}	1.75± 0.49^{ab}
Liver	1.12± 0.2^{b}	1.39± 0.4^{ab}	0.79± 0.2^{b}	0.98± 0.19^{b}	1.34± 0.5^{ab}	1.88± 0.46^{a}
Gill	0.48± 0.52^{c}	1.35± 0.31^{a}	1.17± 0.63^{a}	1.42± 0.62^{a}	0.94± 0.47^{ab}	1.29± 0.66^{a}

Values are represented as means ± SEM of three replicates treatments. C: Healthy fish, negative control; T1: Non-immunized rainbow trout challenged with live trophonts of Ichthyophthirius multifiliis; T2: Rainbow trout immunized via irradiated trophonts; T3: Rainbow trout immunized via irradiated trophonts plus alginate nanoparticles; T4: Rainbow trout immunized via formalin inactive trophonts; T5: Rainbow trout immunized via formalin inactive trophonts plus alginate nanoparticles.

Table 4. Expression of IgM gene in the liver, head kidney and gill of treated juvenile rainbow trout against Ichthyophthirius multifiliis relative to the β-actin gene during 30 days. Different lower case letters (a–c) within the same row (different tissues) indicate significant differences (P< 0.05).

Treatments / Tissues	C	T1	T2	T3	T4	T5
Head kidney	3.2± 0.89^{b}	3.14± 0.38^{b}	6.2± 1.7^{a}	6.06± 1.6^{a}	6.06 ± 1.01^{a}	6.38± 1.66^{a}
Liver	2.01± 0.51^{c}	2.86± 1^{abc}	3.69± 1.2^{ab}	4.27± 1.4^{a}	3.02± 1.07^{abc}	3.84± 1.14^{ab}
Gill	1.49± 0.46^{b}	1.26± 0.41^{b}	3.64± 0.5^{a}	4.27± 1.2^{a}	1.07± 0.35^{b}	3.69± 0.47^{a}

Values are represented as means ± SEM of three replicates treatments. C: Healthy fish, negative control; T1: Non-immunized rainbow trout challenged with live trophonts of Ichthyophthirius multifiliis; T2: Rainbow trout immunized via gamma-irradiated trophonts; T3: Rainbow trout immunized via gamma-irradiated trophonts plus alginate nanoparticles; T4: Rainbow trout immunized via formalin inactive trophonts; T5: Rainbow trout immunized via formalin inactive trophonts plus alginate nanoparticles.

DISCUSSION

In this study, non-immunized fish (T1) challenged with live trophonts showed infection and died. This is similar to results of study of infected *Nile Tilapia* and *Basa fish* (*Pangasius bocourti*) that showed few days to death [21, 22]. The immunized fish with gamma-irradiated and formalin inactive trophonts with/without alginate nanoparticles exhibited light infection with high survival rate (%). The survival rate (%) observed in this study was similar to the

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other fish immunization trials that trophonts provided survival to live trophonts challenge in immunized fish compared to the non-immunized control [23, 24, 25, 26, 27].

Rainbow trout reacted to *I. multifiliis* by upgrading innate and adaptive immune genes to express at the mucosal immune surfaces and in the central immune organs [28, 15]. *IL-8* is an important Cys-Xaa-Cys (CXC) subgroup of chemokines, and also a potent chemoattractant for neutrophils in local inflammation or injury sites [29, 30]. The increase of *IL-8* gene expression in local infection sites post-parasite infection were observed for *I. multifiliis* and *C. goldsmidi* in infection models; This could be an important factor in the extravasations and recruitment of neutrophils into the skin and gill, respectively [6, 31,32]. Skin and gill as mucosal organs can serve as the first line of defense against *I. mutifiliis*. Our previous studies have shown that *IL-8* was up-regulated in the skin of rainbow trout immunized with gamma-irradiated and formalin inactive trophonts of *I. multifiliis* plus alginate nanoparticles [16]. The results obtained from the current study were in line with the report of Heidarieh *et al.* (2015) that showed markedly *IL -8* gene expression upregulated in the head kidney, liver and gill tissues after fish treating with gamma-irradiated with/without alginate nanoparticles and formalin inactive trophonts plus alginate nanoparticles, which was further confirmed by newly reported on upregulating of the cytokines gene expression in both of the systemic and mucosal tissues of vaccinated catfish [15,28]. It was also found that *IL-8* was upregulated in the liver, head kidney and gill of juvenile rainbow trout after infection with live *I. multifiliis*. Previous studies on catfish reported that the expression of cytokines increased in the head kidney, spleen and liver post *I. multifiliis*-infection [34, 33].

In this study, the expression profiles of *MHC-II* and *IL-8* were similar, and both were significantly upregulated in the gill, liver and head kidney of juvenile rainbow trout at most time points after immunization against *I. multifiliis*. The *MHCs* are immunoglobulin superfamily member proteins that interact with T-cells [35]. *MHC-II* mainly presents external peptides (Ags) to cell mediated immunity [36]. These results suggested that renal and gill *MHC-II* gene related to the cell receptors were actively involved in the immune response to combat *I. multifiliis* in juvenile rainbow trout. Our results are also in agreement with the study on the expression of *MHC-II* in *fish* [13] which showed these cell receptor genes were up-regulated in the head kidney of immunized fish from 4 hrs to 10 days after immunization. An up-regulation of *TCR-β* and *MHC-II* genes were also demonstrated following experimental immunization of rainbow trout with a DNA vaccine against *I. multifiliis* [37]. In our studies on juvenile rainbow trout also found that the expression of *MHC-II* genes was significantly unregulated in skin [16] and gill and head kidney after the bath-challenge with live *I. multifiliis* trophonts. Similar results of *MHC-II* were detected in the head kidney of rainbow trout post-infection with *I. multifiliis* theronts [5, 6].

Serum and mucosal antibodies were detected in fish immunized with live theronts [38]. The antibody repertoires in teleost fish are more limited than in mammals [39]. The most prevalent immunoglobulin in serum of fish is an *IgM* gene [30]. IgM mainly functions in the adaptive immune response in the systemic tissues of rainbow trout [15]. *IgM* gene up-regulation was also reported by Olsen *et al.* (2011) in trout gill following immunization by IP injection of *I. multifiliis* theronts [40]. We here demonstrated a significant up-regulation of *IgM* gene in the gill and head kidney tissues following treatment with gamma-irradiated and formalin inactive trophonts with/without alginate nanoparticles at days 30 post-immunization in agreement with other reports [6]. A previous experiment also showed an up-regulation of *IgM* in the skin of the rainbow trout from day 20 to day 30 after immunization with gamma-irradiated and formalin-inactive trophonts plus alginate nanoparticles [16]. These results are indicators of production of antibodies in gill as local mucosal surface and head kidney as central immune organs of immunized rainbow trout against *I. multifliis*. Sigh *et al.* (*2004*) reported rainbow trout contained high titers of parasite-specific *IgM* in the serum in the head kidney after

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I. multifiliis infection [5]. According to the obtained results, *IgM* might be generated in the head kidney and transported to other lymphoid organs by blood circulation [23].

Formalin inactive trophonts as well as gamma-irradiated trophonts successfully can induce antibody-mediated protection rainbow trout, but use of gamma radiation method can abolish chemical contaminants and penetrates pathogens to destroy nucleic acids without damaging the pathogen surface antigens. Moreover, there is no need to remove any chemical residue after inactivation. Although some gamma-irradiated trophonts showed no surprising results compared to live attenuated- or chemically inactivated vaccine antigen, the demand for the application of gamma radiation is increasing for the development of the safe vaccine for fish in aquaculture [41, 42, 43].

Furthermore, this study focused on the expression of some genes (*IgM, MHC-II and IL-8*) related to innate and adaptive immune responses in the liver, head kidney (as central immune organs) and gill (as mucosal immune surface) tissues of non-immunized and immunized rainbow trout with gamma-irradiated and formalin inactive trophonts plus alginate nanoparticles as adjuvant against *I. multifiliis*. The acquired results showed the entire treatments can have positive effects on both antibody and cell mediated immune responses at local and systemic level as seen by gene expression in all examined tissues.

***Conflicts of interest*:** *Declared none.*

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