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Research paper

Protec™ improves innate immune response and specific antibody response against *Lactococcus garvieae* in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Protec™ is a commercial aquafeed (Skretting Italia) containing a combination of glucans, vitamin C, vitamin E and zinc (immune support pack). No research information concerning its capability to improve fish immune response is available, so in this study the potential immunomodulatory effects of Protec™ were investigated in rainbow trout (*Oncorhynchus mykiss*). Head kidney (HK) leukocytes from adult fish (100 g, n = 6) were *in vitro* incubated with Protec™ immune support pack resulting in significantly higher respiratory burst activity and proliferation. Specifically, sonicated Protec™ immune support pack (160 µg/ml) induced a respiratory burst response similar to that promoted by zymosan and lipopolysaccharide (LPS), while non-sonicated Protec™ immune support pack induced a response comparable to that of cells stimulated with phorbol myristate acetate (PMA). Moreover, the proliferation of leukocytes exposed to sonicated Protec™ immune support pack (20 µg/ml) was significantly higher than that of cells stimulated with zymosan, and it was comparable to the proliferation of cells stimulated with phytohaemagglutinin (PHA) and LPS. Afterwards, a feeding trial was performed in a rainbow trout farm. Two groups of juvenile rainbow trout (10 g) were acclimated for 7 weeks before the experiment and fed daily with a commercial control diet (Optiline HE, Skretting Italia) at 2% BW/day. At the end of acclimation, one group of fish was fed with Protec™ diet (Skretting Italia) at 2% BW/day whereas the other group continued to feed the control diet at the same level for further 4 weeks. Then, fish were sampled (HK leukocytes from n = 6 fish/group, serum from n = 12 fish/group) or intraperitoneally vaccinated against lactococcosis (n = 160/dietary group/time point). Fish fed the same diets for further 4 weeks after vaccination, then feeding returned to the control diet in both groups until the end of the trial. The specific antibody response was recorded at 4 and 8 weeks after vaccination (n = 12 fish/group). The administration of Protec™ significantly enhanced the respiratory burst activity of leukocytes and the synthesis of specific IgM against *Lactococcus garvieae*, whereas the serum lysozyme activity was unaffected. The present research suggests that the administration of Protec™ can improve both innate and adaptive immune response of rainbow trout, proving to be an interesting strategy for enhancing the immune reactivity of fish to vaccines.

1. Introduction

Worldwide aquaculture has experienced a strong intensification over the past decades and this aspect is correlated to a higher probability for disease outbreaks caused by both bacterial and viral pathogens. These episodes are often responsible for high mortality rates and decreased productivity, leading to severe economic losses for fish farmers.

The use of immunostimulants is an environmental-friendly and cost-effective approach to ensure the health status of reared fish, as they are known to enhance innate immunity and provide sufficient protection against a range of fish pathogens (Wang et al., 2017). Classical immunostimulants are biologically intended as PAMPs and bind to specific PRRs on the surface of cells such as monocyte-macrophages and granulocytes, triggering the *target* cells activation towards pathogens and leading to the release of pro-inflammatory cytokines which mediate the

Abbreviations: CL, chemiluminescence; COX-2, cyclooxygenase-2 HK, head kidney; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; RB, respiratory burst; RLU, relative luminescence units; ROS, reactive oxygen species; RT, room temperature; SSA, serum amyloid a

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onset of the innate immune responses (Chettri et al., 2010; Fierro-Castro et al., 2012). Moreover, alternative dietary factors like amino acids, fatty acids, minerals and vitamins could affect human and animal immune responses (Trichet, 2010).

Initial screening of immunostimulants in fish can be preliminarily achieved by studying *in vitro* the interaction of leucocytes with the candidate substances, so as to reduce the costs of analyses as well as the number and pain of experimental animals (Directive, 2010/63/EU). In this regard, whole purified leukocytes isolated from fish hematopoietic organs and/or cell cultures consisting of single populations have been successfully used to study the immunostimulatory properties of glucans, LPS, zymosan, vitamins, seaweed derivatives or medicinal plant extracts (Mulero et al., 1998; Castro et al., 1999, 2006; Chettri et al., 2010, 2011; Abarca et al., 2012; Fierro-Castro et al., 2012; Bulfon et al., 2018). Some authors used primary tissue cultures (*i.e.* explants from the spleen) for such purposes, since they may reflect better the *in vivo* responses compared to cultures of single cell populations. However, such kinds of primary cell cultures cannot easily be standardized, and there is a continual need for fish stocks to establish explant cultures for new experiments (Fierro-Castro et al., 2012).

If possible and in accordance with the cardinal principle of the 3Rs ("Replacement, Reduction and Refinement") that should be applied for animal welfare (Midtlyng et al., 2011; Romberg et al., 2012), *in vitro* tests should be followed by *in vivo* experiments to elucidate whether the benefits of immunostimulants also occur in living fish. The dietary administration is the most applied approach selected for research studies as it represents the method more economically suited to extensive aquaculture, being not stressful and allowing a mass administration regardless of fish size (Wang et al., 2017).

The present study was designed to experimentally assess the efficacy of the commercial Protec™ aquafeed (Skretting Italia), containing a combination of glucans, vitamin C, vitamin E and zinc (immune support pack), in enhancing fish immune response. Protec™ aquafeed is commonly applied both in freshwater and in seawater fish farming. However, no scientific studies investigating its immunostimulant effects in aquatic organisms have been published so far. For this reason, a dual approach was followed, including a preliminary *in vitro* examination of the immunomodulatory properties of the Protec™ immune support pack on respiratory burst activity and proliferation of rainbow trout (*Oncorhynchus mykiss*) HK leukocytes. Afterwards, an *in vivo* feeding trial was performed to evaluate the effect of Protec™ administration on the innate immune response of rainbow trout and on the synthesis of specific antibodies after a vaccination against lactoccoccosis.

2. Materials and methods

2.1. Reagents

FCS; gelatine; HBSS, Hank's Balanced Salt Solution without phenol red, Ca2+ and Mg2+; heparin (5 KU/ml); Histopaque®1077; L-15, Leibovitz medium; LPS; L-glutamine; lysozyme from chicken egg white; luminol, 5-amino-2,3-dihydro-1,4-pathalazinedione; *Micrococcus lysodeikticus*; MS-222, tricaine methane sulfonate; MTT, 3 - [4, 5 - dimethylthiazol-2 yl] - 2, 5 - diphenyl tetrazolium bromide; penicillin/streptomycin solution; PHA; PMA; poly-L-lysine; TMB, 3,3′,5,5′-tetramethylbenzidine hydrochloride; trypan blue; zymosan were purchased from Sigma-Aldrich (Milan, Italy). All other chemicals were of reagent grade.

2.2. In vitro evaluation

2.2.1. Protec™ immune support pack preparation

An aliquot of Protec™ immune support pack (active components provided by Skretting Italia and conventionally incorporated in the Protec™ aqua feed) was prepared in bidistilled water (1 mg/ml) and submitted to sonication by HielSheker UP400S sonar, at frequency 24 KHz and 400 W power, at +4 °C for 30 min. A second aliquot was not submitted to sonication. Afterwards, both suspensions were sterilized by autoclave in order to exclude a possible microbiological contamination and stored at +4 °C until use.

2.2.2. Leukocyte purification

Healthy female rainbow trout (average body weight 100 g) were obtained from a local fish farm and reared in the facilities of Udine University (DI4A) in squared fiberglass tanks in an indoor open system supplied with filtered freshwater (water renewal = 0.25 l/s per tank) and fed daily with a commercial extruded diet (Optiline HE, Skretting Italia) at 1% BW/day in two meals administered at 9:00 and 16:00 h 6 days per week. The physicochemical parameters of water (temperature 14.5 °C, pH 8.0, dissolved oxygen 9.5 mg/l, NH_4-N 0.02–0.03 mg/l, NO_2-N < 0.015 mg/l, 12 h light/12 h dark period) were maintained throughout the experimental phase. The purification of HK leukocytes was performed as previously described by Bulfon et al. (2018), after fish sacrifice with an overdose of MS-222 (400 mg/l). Briefly, the organ was aseptically pressed in HBSS with 0.25% heparin and the cell suspension was layered on Histopaque®1077, then centrifuged at 300x*g* for 25 min at 7 °C. Leukocytes at the interface were washed twice and adjusted to 1×10^7 cells/ml in L-15 containing 2 mM L-glutamine, 10% FCS, penicillin 100 U/ml and streptomycin 100 µg/ml (culture medium) or in HBSS, and kept on ice until assay performance (maximum 10 min). Cells from 6 fish/parameter were maintained as individual cultures throughout the experiments and tested in duplicates on the same sampling day.

2.2.3. Leukocyte respiratory burst activity

The RB activity was assessed using a CL assay into sterile black 96-well plates (Nunc, Sigma Aldrich), according to the method of Coteur et al. (2002), with minor modifications. Leukocytes (100 µl/well) in HBSS were incubated with 50 µl/well luminol (2 mM) and 100 µl/well Protec™ immune support pack, LPS, zymosan at 160 µg/ml or PMA (2.5 µg/ml) as positive control. PMA concentration was chosen based on author experience whereas the concentration of the other immunostimulants was selected based on preliminary experiments in which rainbow trout leukocytes were exposed to Protec™ immune support pack (sonicated or non-sonicated) at concentrations ranging between 10 µg/ml and 160 µg/ml. In this context, the concentration equal to 160 µg/ml induced the strongest cell stimulation, compared to non-stimulated cells (Fig. 1A and B). Wells without stimulus were included as blank. The CL emission was measured as RLU using a luminometer (Tecan S.r.l., Milan, Italy), starting from 3 min after adding the stimulus, every 3 min for 15 min and subsequently every 5 min for 45 min at RT. The RB activity was expressed as RLU/1×10^7 cells/ml.

2.2.4. Leukocyte proliferation

Leukocytes in culture medium (100 µl/well) were distributed into sterile flat-bottom 96-well plates (Sarstedt, VR, Italy) and exposed to 100 µl/well Protec™ immune support pack (sonicated and non-sonicated), LPS, zymosan at 20 µg/ml and 160 µg/ml or PHA (10 µg/ml) as positive control. PHA concentration was selected based on author experience whereas the concentrations of the other immunostimulants were chosen in order to be comparable to that used for respiratory burst evaluation. Leukocytes incubated only with culture medium were included as negative control. After 72 h incubation at 18 ± 2 °C, the microplates were centrifuged (300x*g* for 10 min at 18 °C) and the supernatant was discarded. The proliferation was measured by the indirect colorimetric MTT based assay (Galeotti et al., 1999).

2.3. In vivo evaluation

2.3.1. Fish and experimental design

Rainbow trout juveniles with an average body weight of 10 g were maintained in a local fish farm into two cement basins receiving a

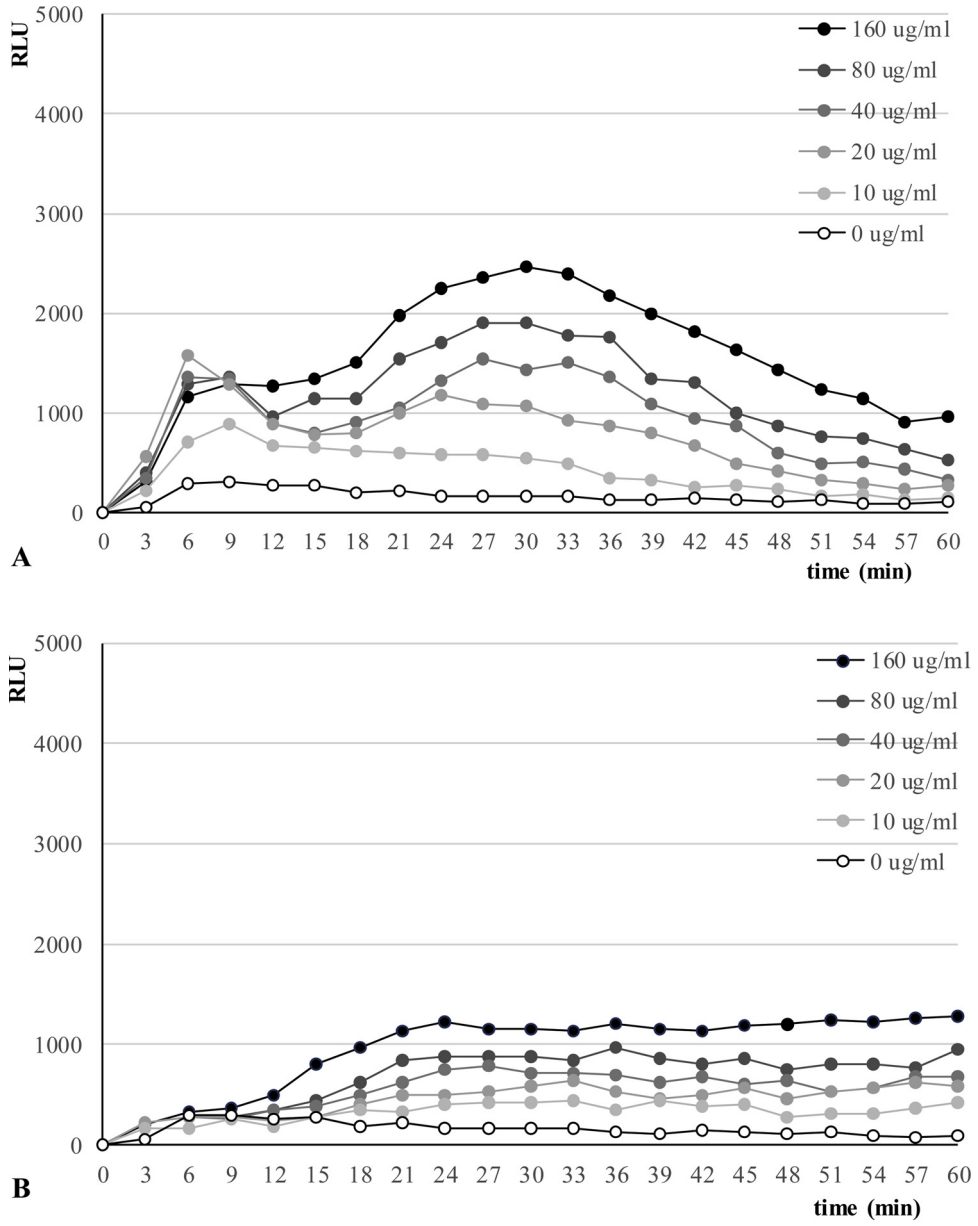

Fig. 1. Respiratory burst activity (kinetic response) of rainbow trout HK leukocytes exposed to non-sonicated (A) and sonicated Protec™ immune support pack (B) at different concentrations. Data (RLU/10^7 cells/ml) are referred to a pilot experiment preliminarily performed on leukocytes purified from the HK of one rainbow trout, to select the best concentration of product to be used in the subsequent *in vitro* assays.

constant water flow from a river at an average temperature of 14 °C under natural photoperiod. Water quality conditions (pH 7.0–8.0, dissolved oxygen 9.5 mg/l, NH_4-N 0.02–0.03 mg/l, NO_2-N < 0.015 mg/l) were monitored during the trial and kept at levels suitable to trout farming. Fish were acclimated for 7 weeks before the experiment, maintained at a density of 0.1 kg/m^3 and fed daily with a commercial control diet without supplementary ingredients (Optiline HE, Skretting Italia) at 2% BW/day. At the end of acclimation, one group of fish was fed with Protec™ diet (Skretting Italia) at 2% BW/day for 4 weeks whereas the other group continued to feed the control diet at the same level for 4 weeks. Then, fish were submitted to a 24 h fasting and sampled for serum and HK (pre-vaccination sampling). The remaining trout were vaccinated against lactococcosis by using Lacto-Fish Vax formulation (Fatro, Italy). Prior to the vaccination, fish were removed from the farming basins and anaesthetized in oxygenated water containing MS-222 (100 mg/l). Then, they were i.p. vaccinated (0.1 ml/fish) and immediately returned to the original basin where they recovered from anesthesia. Fish fed the same diets (Protec™ and control) for further 4 weeks after vaccination, then feeding returned to the control diet in both groups until the end of the trial. Fish were kept under constant veterinary control and no clinical sign of disease or mortality were registered.

2.3.2. Sampling

After the first 4 weeks-feeding with Protec™ diet, 12 randomly selected fish for each dietary treatment were anaesthetized with MS-222 and submitted to blood collection from the caudal vein. Samples were kept in ice. Serum was obtained after centrifugation at 1500x*g* for 15 min at 4 °C and stored at −80 °C until the quantification of lysozyme activity. After blood sampling, 6 fish/group were sacrificed by an overdose of anesthetic and HK was aseptically removed, then processed as already described above for the *in vitro* assays. Fish left in the experimental basins were subsequently submitted to blood collection at 4 and 8 weeks after vaccination (n = 12 fish/group/sampling), to

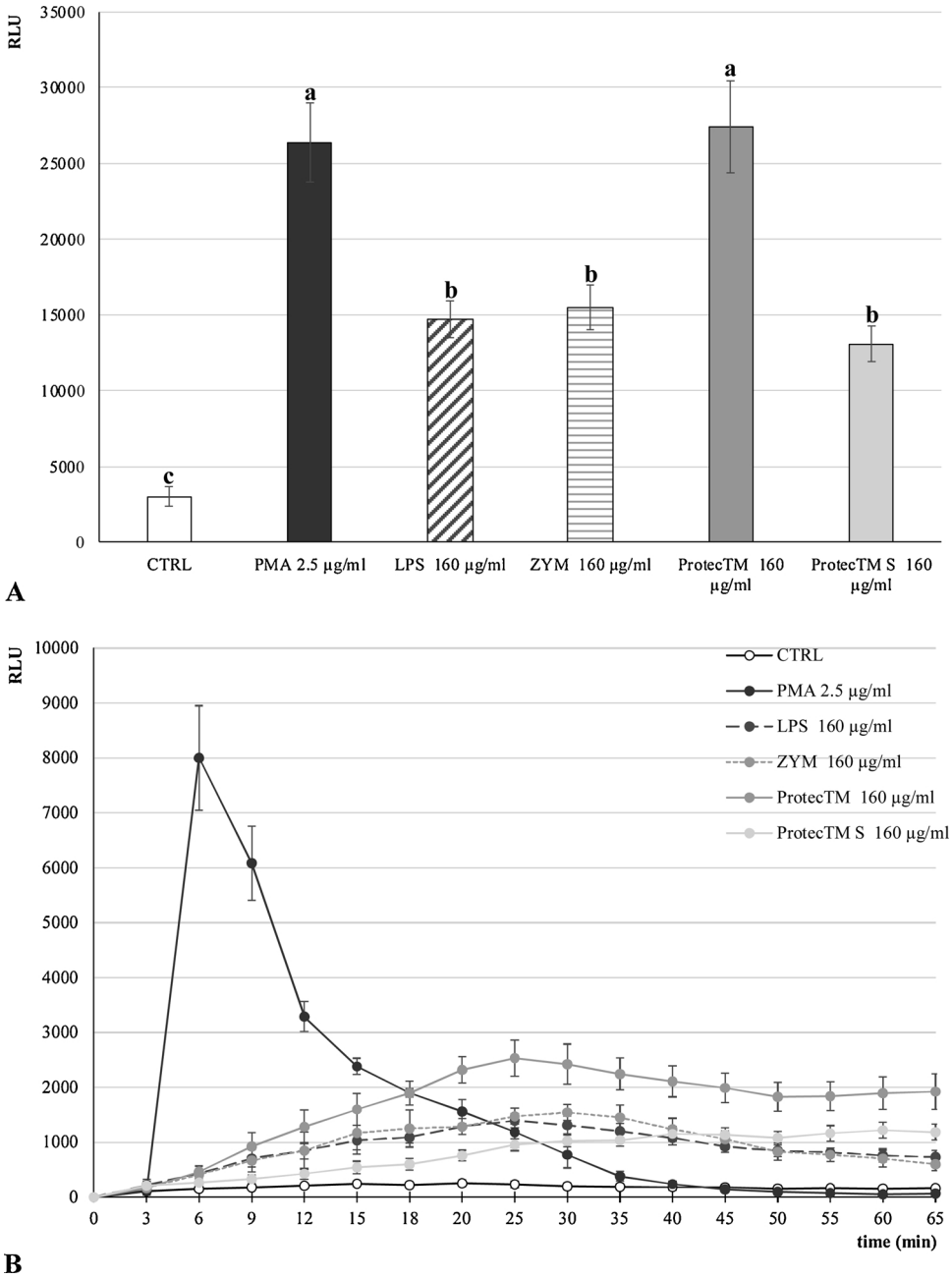

Fig. 2. Respiratory burst activity (A, cumulative response; B, kinetic response) of rainbow trout HK leukocytes exposed to non-sonicated and sonicated Protec™ immune support pack (Protec™ TM and PrtecTM and Protec™, respectively), LPS, zymosan (ZYM) and PMA or not stimulated (CTRL). Data (RLU/10^7 cells/ml) are expressed as mean ± SEM from n = 6 independent fish (in duplicate). Different letters indicate significant differences among different stimuli ($P \leq 0.05$).

quantify the specific antibody titre against *Lactococcus garvieae* in the serum.

2.3.3. Innate immune response

The serum lysozyme activity was determined according to Parry et al. (1965). Briefly, 10 µl of serum were incubated in 96-well microtiter plates (Sarstedt, VR, Italy) with 200 µl of 0.02% lyophilized *M. lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.8). The reduction of absorbance was measured at 450 nm every 10 min for 1 h using a microplate reader (Sunrise, Tecan S.r.l., MI, Italy). The lysozyme activity (U/ml) was calculated from a standard curve prepared with serial dilutions of chicken egg white lysozyme.

At the same time, the RB activity of HK leukocytes was quantified by the method already performed for the *in vitro* assays, using leukocytes suspended in HBSS at 10^7 cells/ml and stimulated with PMA (2.5 µg/ml). The RB activity was expressed as cumulative response (RLU/10^7 cells/ml).

2.3.4. Adaptive immune response

The specific anti-*L. garvieae* IgM titre was detected by and indirect ELISA accordingly to Galeotti et al. (2013), with minor modifications. Poly-L-lysine treated microplates (Maxisorp, Sigma Aldrich) were coated with formalin inactivated *L. garvieae* bacterin (kindly provided by Dr. Prearo, IZS Piemonte, Liguria e Valle d'Aosta) for 1 h at RT (100 µl/well). The bacterial concentration used for plate coating was estimated by reading the OD at 610 nm and by adjusting it to 1.0 with sterile PBS (corresponding to 1×10^9 bacteria/ml). Then, the plates were blocked with 1% gelatine for 3 h at RT and 5% goat normal serum overnight. Twofold serial dilutions (in duplicate) of sera from 12 fish/group were incubated for 3 h at RT. Negative controls (serum omission

or non-immunized rainbow trout serum) and positive control (*L. garvieae* immunized rainbow trout serum) were included. A mouse mAb against rainbow trout IgM (Aquatic Diagnostic Ltd., UK) was added (dil. 1:33, 100 µl/well) for 1 h at RT, followed by HRP-labelled goat anti-mouse IgG (Sigma-Aldrich) (1:1500, 100 µl/well) for 1 h at RT. The reaction was revealed using as substrate 0.42 mM TMB and 0.001% H_2O_2, then stopped with 2 M H_2SO_4 (50 µl/well). The OD was read at 450 nm with an automatic plate reader.

The agglutination antibody titre was evaluated in round-bottomed microplates (Sarstedt) according to Barnes and Ellis (2004) with minor modifications. Fifty µl/well of two-fold dilutions of serum in PBS (pure, 1/2, 1/4, 1/8, 1/16, and 1/32) were incubated with an equal volume of *L. garvieae* bacterin (OD of bacterial suspension at 620 nm = 0.3) for 24 h at 22 ± 2 °C. The agglutination antibody titre was calculated and expressed as log2 of reciprocal of the highest serum dilution that gives bacterial agglutination, as described by Yarahmadi et al. (2014).

2.4. Statistical analyses

The results are expressed as arithmetic mean ± SEM. Statistical analyses were performed using the SPSS version 20.0, Inc. Chicago, IL, USA. Data were tested for normality using the Kolmogorov-Smirnov test. The results of *in vitro* assays were analysed by one-way analysis of variance (ANOVA) and Duncan's *post hoc* test for multiple comparisons. If the variances were not normally distributed, the Kruskal–Wallis non-parametric test and the relative Mann-Whitney *U post hoc* test were applied. The results of the *in vivo* trial were opportunely analysed by the *t* Student test or Mann-Whitney *U* test (non-parametric) to detect significant differences in the lysozyme activity, respiratory burst activity and antibody level between the two dietary treatments or in the antibody level between the two sampling times after vaccination. Significance level was set to $P \leq 0.05$.

3. Results

3.1. In vitro evaluation

3.1.1. Leukocyte respiratory burst activity

The RB (Fig. 2A) of stimulated leukocytes was significantly higher than that of control cells ($P \leq 0.05$). In particular, non-sonicated Protec™ immune support pack at concentration of 160 µg/ml induced a RB activity significantly higher than that measured in control cells, and in cells incubated with 160 µg/ml LPS, zymosan and sonicated Protec™ immune support pack ($P \leq 0.05$). This response was comparable to that of leukocytes stimulated with 2.5 µg/ml PMA ($P > 0.05$). Sonicated Protec™ immune support pack at concentration of 160 µg/ml induced a significantly higher oxidative response than that detected in control cells ($P \leq 0.05$). It was comparable to the ROS production induced by 160 µg/ml LPS and zymosan ($P > 0.05$) but it was significantly lower to the response induced by PMA ($P \leq 0.05$).

The RB activity of cells stimulated with the immune support pack of Protec™ showed a kinetic similar to that induced by LPS and zymosan, which was postponed compared to that induced by PMA. In fact, the maximum ROS production in leukocytes stimulated with Protec™ immune support pack, LPS and zymosan was recorded after 25–60 min of incubation whereas the ROS production induced by PMA showed a maximum peak after six min (Fig. 2B).

3.1.2. Leukocyte proliferation

The proliferation of leukocytes is shown in Fig. 3. Sonicated Protec™ immune support pack at concentration of 20 µg/ml stimulated the proliferation of leukocytes compared to control cells, with a comparable intensity to that induced by 20 µg/ml PHA, LPS and non-sonicated Protec™ immune support pack ($P > 0.05$) but with a significantly higher intensity than 20 µg/ml zymosan ($P \leq 0.05$). There were no significant differences between the proliferation of cells incubated with 20 µg/ml non-sonicated Protec™ immune support pack and that of untreated cells ($P > 0.05$). The proliferation of leukocytes exposed to sonicated and non-sonicated Protec™ immune support pack at concentration of 160 µg/ml was significantly lower compared to the response of cells stimulated with PHA ($P \leq 0.05$) and similar to that of control cells and leukocytes stimulated with 160 µg/ml LPS and zymosan ($P > 0.05$).

3.2. In vivo evaluation

3.2.1. Innate immune response

The serum lysozyme activity was not significantly modulated by the administration of Protec™ diet ($P > 0.05$) (Fig. 4A). On the contrary, the leukocyte RB activity in rainbow trout fed Protec™ for 4 weeks was significantly improved compared to that measured in the control group ($P \leq 0.05$) (Fig. 4B).

3.2.2. Adaptive immune response

The post vaccination specific humoral response against *L. garvieae* is illustrated in Fig. 5A. In general, vaccination stimulated a scarce antibody synthesis (low OD values) in rainbow trout as detected by indirect ELISA, if compared with the positive control serum showing an OD of about 0.5. However, a significant enhancement of anti-*L. garvieae* IgM level was detected in fish fed Protec™, both at 4 and 8 weeks after i.p. vaccination ($P \leq 0.05$). Moreover, the specific antibody titre in the control group significantly decreased 8 weeks after immunization ($P \leq 0.05$) compared to the antibody response measured after 4 weeks, whereas the specific IgM level in fish fed Protec™ persisted for a longer time so that it resulted similar at 4 and 8 weeks after vaccination ($P > 0.05$).

No significant differences were observed in the antibody agglutination capacity upon vaccination between fish fed the control diet and fish fed Protec™ ($P > 0.05$) (Fig. 5B).

4. Discussion

In the present research, a dual approach including *in vitro* and *in vivo* insights was followed to study if the commercial Protec™ aquafeed, containing a combination of glucans, vitamin C, vitamin E and zinc (immune support pack), can effectively stimulate the innate and acquired immune responses of rainbow trout (*O. mykiss*), which was selected as a model species due to its importance in Italian freshwater aquaculture. To date, no scientific studies investigating its efficacy in aquatic organisms have been published.

The effects of Protec™ immune support pack were preliminarily *in vitro* studied on rainbow trout HK leucocyte responses. In this case, the activation of cells exposed to Protec™ immune support pack was compared to that induced by other known immunostimulants such as LPS and zymosan. We decided to test both sonicated and non-sonicated Protec™ immune support pack to detect eventual differences of *in vitro* immunostimulant properties between the two forms, and then to compare the *in vitro* results with the in *vivo* responses as regards the non-sonicated form which is exclusively included in the commercial aquafeed.

Experiments proved that sonicated immune support pack of Protec™ (160 µg/ml) stimulates the respiratory burst activity of rainbow trout leukocytes likewise to LPS and zymosan, whereas non-sonicated Protec™ immune support pack induces a much higher cumulative response, which is comparable to that induced by PMA. In general, the kinetic of ROS production in cells stimulated with both the formulations of Protec™ immune support pack (sonicated and non-sonicated) was similar to that induced by LPS and zymosan, and it developed later than that induced by PMA.

It is well known that fish HK leukocytes have the ability to recognize several PAMPs mimicking diverse pathogen types, thus eliciting differential responses and the expression of various immune genes. Our

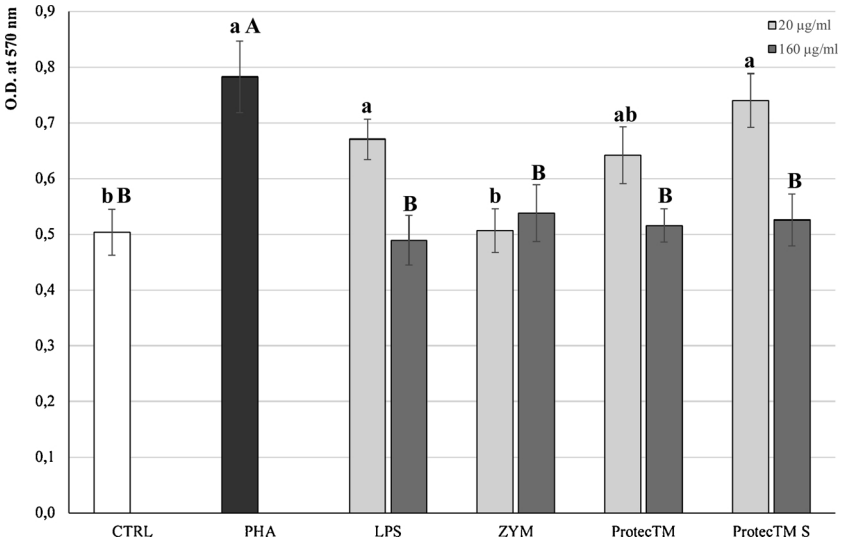

Fig. 3. Proliferation of rainbow trout HK leukocytes exposed to non-sonicated and sonicated Protec™ immune support pack (Protec™ and Protec™ S, respectively), LPS, zymosan (ZYM) and PHA or not stimulated (CTRL). Data (OD at 570 nm) are expressed as mean ± SEM from n = 6 independent fish (in duplicate). Different letters (small letters are referred to 20 µg/ml; capital letters are referred to 160 µg/ml) indicate significant differences among different stimuli (P ≤ 0.05).

results suggest that the activation of respiratory burst depends mainly on the presence of PAMPs in the Protec™ immune support pack, such as those expressed by glucans. Glucans are known to stimulate the respiratory burst in fish through a mechanism of PAMP-membrane PRR interaction (Vera-Jimenez et al., 2013), rather than a mechanism of absorption similar to that of PMA or other soluble compounds that rapidly enter the cell (Tsumbu et al., 2012). Our observations confirmed the results already provided by several authors, which demonstrated that glucans, LPS and zymosan (which also contains beta glucans along with outer cell wall components of fungi) strongly stimulate the respiratory burst activity of rainbow trout leukocytes (Chettri et al., 2010, 2011; Abarca et al., 2012; Fierro-Castro et al., 2012). This response has been proved to be associated with increased mRNA transcripts of IL-1ß, IL-6, TNF-α, IL-10 and COX-2 (Chettri et al., 2011; Fierro-Castro et al., 2013). In addition, vitamin C has been reported to increase the production of ROS (Verlhac et al., 1996; Leal et al., 2017), the percentage of phagocytic cells and the transcription of several pro-inflammatory and antimicrobial genes (Leal et al., 2017) in rainbow trout HK leukocytes.

In the present study, the sonicated immune support pack of Protec™ (20 µg/ml) also acted as mitogen by improving the proliferation of leukocytes as observed in cells stimulated with LPS and PHA, but more effectively than zymosan. Various yeast beta glucans have been defined as T-cell oriented immunopotentiators, functioning as maturation factors of lymphoid cells in mammals (Verlhac et al., 1998). We can hypothesize that glucans included in Protec™ modulate the proliferative response of rainbow trout leukocytes with a similar mechanism of action. Interestingly, when used at higher concentration (160 µg/ml) the Protec™ immune support pack did not significantly influence the leukocyte proliferation. In this regard, Castro et al. (1999) previously observed that increasing concentrations of beta glucans stimulated less effectively the leukocyte respiratory burst response in turbot (*Psetta maxima*) and gilthead sea bream (*Sparus aurata*), probably because these immunostimulants at high doses compete for the binding to the receptors present on cell surface and for their subsequent internalisation.

In this research, we used all the leukocytes purified from rainbow trout HK to evaluate the immunomodulatory properties of Protec™ immune support pack because cell culture systems have been considered able to provide more predictive information on *in vivo* responses than cell cultures mainly formed by a single cell population (Fierro-Castro et al., 2012). However, further studies are needed to elucidate which population(s) of leukocytes is more effectively activated in response to the active components of Protec™ aquafeed. Moreover, new investigation are recommended to explain the effect of sonication and autoclave treatment (heat stability) on the characteristics of Protec™ active components and to understand the differential *in vitro* effects of the two formulations (sonicated and non-sonicated).

In the present study, we also investigated the effects of Protec™ administration on juvenile rainbow trout immune response, highlighting an enhanced leukocyte respiratory burst activity in fish fed Protec™ diet for 4 weeks compared to the control group. This evidence is consistent with the preliminary *in vitro* results, confirming the ability of non-sonicated Protec™ components to promote this important aspecific mechanism of leukocyte defense against pathogens. We can suggest that purified leukocytes are activated in terms of ROS release by direct *in vitro* contact with the active compounds of the aquafeed, but possibly even when these compounds are orally administered to the fish. *In vivo* they are probably able to stimulate the mucosal (GALT) immune responses and consequently the synthesis of cytokines that systemically drive the activation of HK leukocytes. On the contrary, the serum lysozyme activity was not affected by the administration of Protec™.

The importance of glucans in inducing disease resistance has been highlighted in different farmed fish species. In particular, it is well known that the inclusion of beta glucans in the diet of rainbow trout positively modulates various immunological parameters (lysozyme, complement, phagocytosis, myeloperoxidase activity, lymphocyte proliferation) and up-regulates the expression level of some immune-related genes (TFN-α, IL-1β, IL10, COX-2, TGF-β, HSP70, lysozyme) (Verlhac et al., 1998; Siwicki et al., 2004; Ghaedi et al., 2015; Douxfils et al., 2017; Ji et al., 2017). Similarly, essential nutrients such as proteins, essential fatty acids, vitamins, polysaccharides and some minerals have a pivotal importance to reinforce normal immune functions in teleost (Miar et al., 2013). Indeed, a significant increase of oxidative burst, pinocytosis, phagocytosis, and lysozyme activity in rainbow trout fed diets enriched with vitamin C (Verlhac et al., 1998) and vitamin E (Clerton et al., 2001) has been previously reported. Concerning zinc, it is well known that it acts as a co-factor for a large number of proteins and enzymes, and it is essential for phagocytosis, intracellular killing and cytokines production (Gharekhani et al., 2015). However, to date new insight are needed to explore the link between the dietary Zn level and fish immune responses. In this regard, dietary Zn-enriched yeast effectively enhanced serum lysozyme activity, complement activity, total antibody and survival against *Yersinia ruckeri* in rainbow trout (Gharekhani et al., 2015).

The present investigation also offered the opportunity to combine a prolonged Protec™ administration with the application of a common protocol of trout vaccination against lactococcosis. Lactococcosis caused by *Lactococcus garvieae* is one of the most important bacterial diseases affecting rainbow trout and can lead to mortality rates of

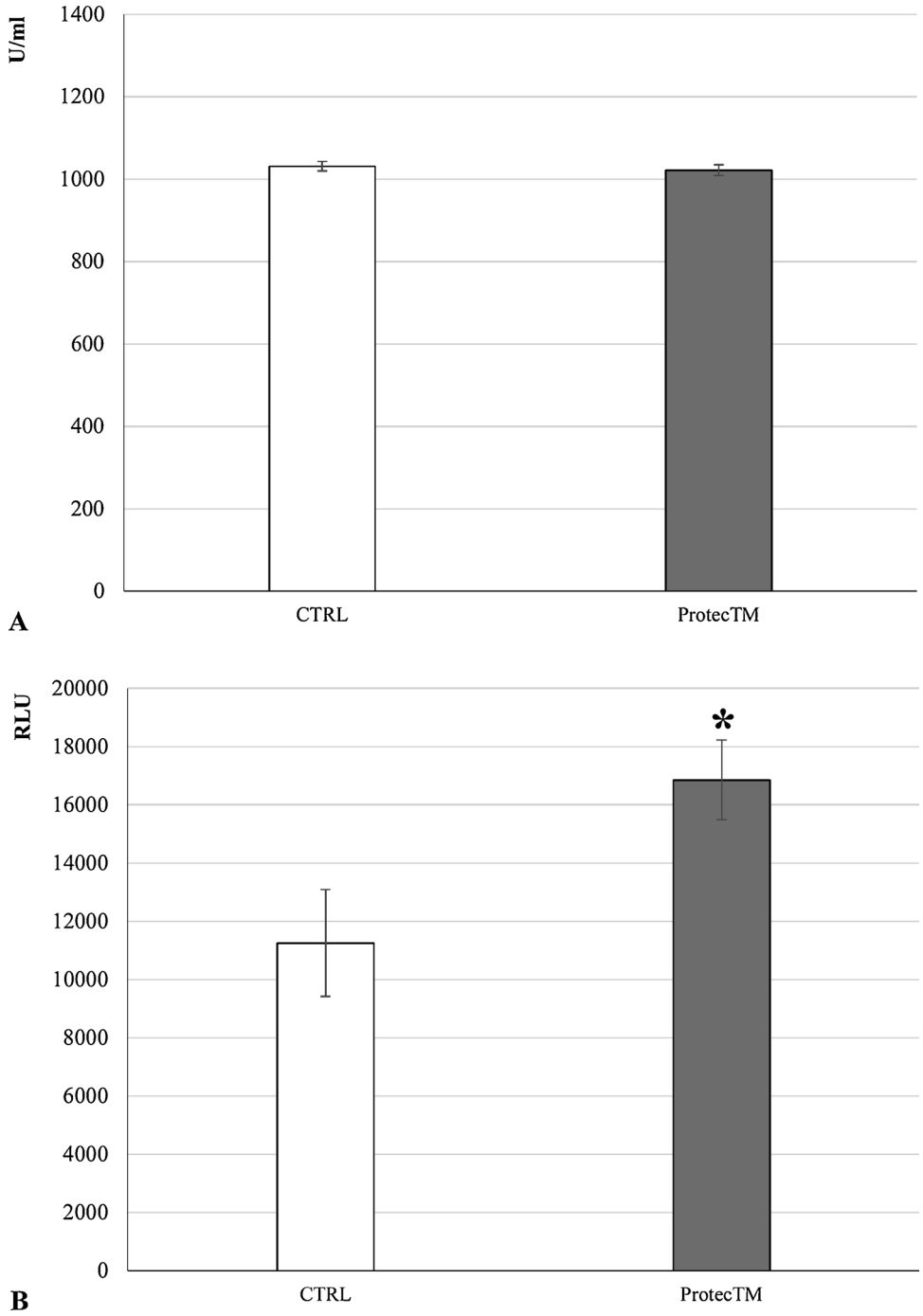

Fig. 4. Lysozyme activity (U/ml) (A) and respiratory burst activity of HK leukocytes (RLU/10^7 cells/ml) (B) in rainbow trout fed Protec™ or control diet for 11 weeks. Data are expressed as mean ± SEM (n = 12 fish for lysozyme evaluation, n = 6 fish for respiratory burst evaluation). The asterisk indicates a significant difference compared to control group ($P \leq 0.05$).

30–50% at the end of the productive cycle, thus causing serious economic losses in intensive farms. Various vaccines have been developed and good protections levels have been achieved only by intraperitoneal protocols (Ceschia et al., 1998; Ravelo et al., 2006; Vendrell et al., 2006, 2007; Kubilay et al., 2008; Bastardo et al., 2012; Tanrikul, 2012).

The possibility to improve the vaccination efficacy in salmonids by pre or post treating the fish with immunostimulants has already been investigated. In this regard, Gioacchini et al. (2008) observed that diets enriched with Ergosan increased the expression levels of IL-1β, IL-8 and TNF-α in juvenile rainbow trout vaccinated against enteric red mouth disease (ERM), improving fish reactivity to vaccination. Likewise, Skov et al. (2012) reported that beta glucans orally administered in ERM vaccinated rainbow trout had a significant effect on the regulation of IL-1ß, SAA, precerebellin and hepcidin expression. Moreover, some authors demonstrated that glucans and vitamin C act as adjuvants by increasing the rainbow trout antibody response if simultaneously administered with a vaccine against *Y. ruckeri* (Veralch et al., 1998; Siwicki et al., 2004).

Our results demonstrated that the administration of Protec™ to fish improves the antibody synthesis after i.p. vaccination against *L. garvieae*, being the specific IgM significantly higher in rainbow trout fed Protec™ than in fish fed the control diet, both at 4 and 8 weeks after the immunization treatment. However, Protec™ did not shown significant effects on antibody agglutination capacity. This evidence could suggest

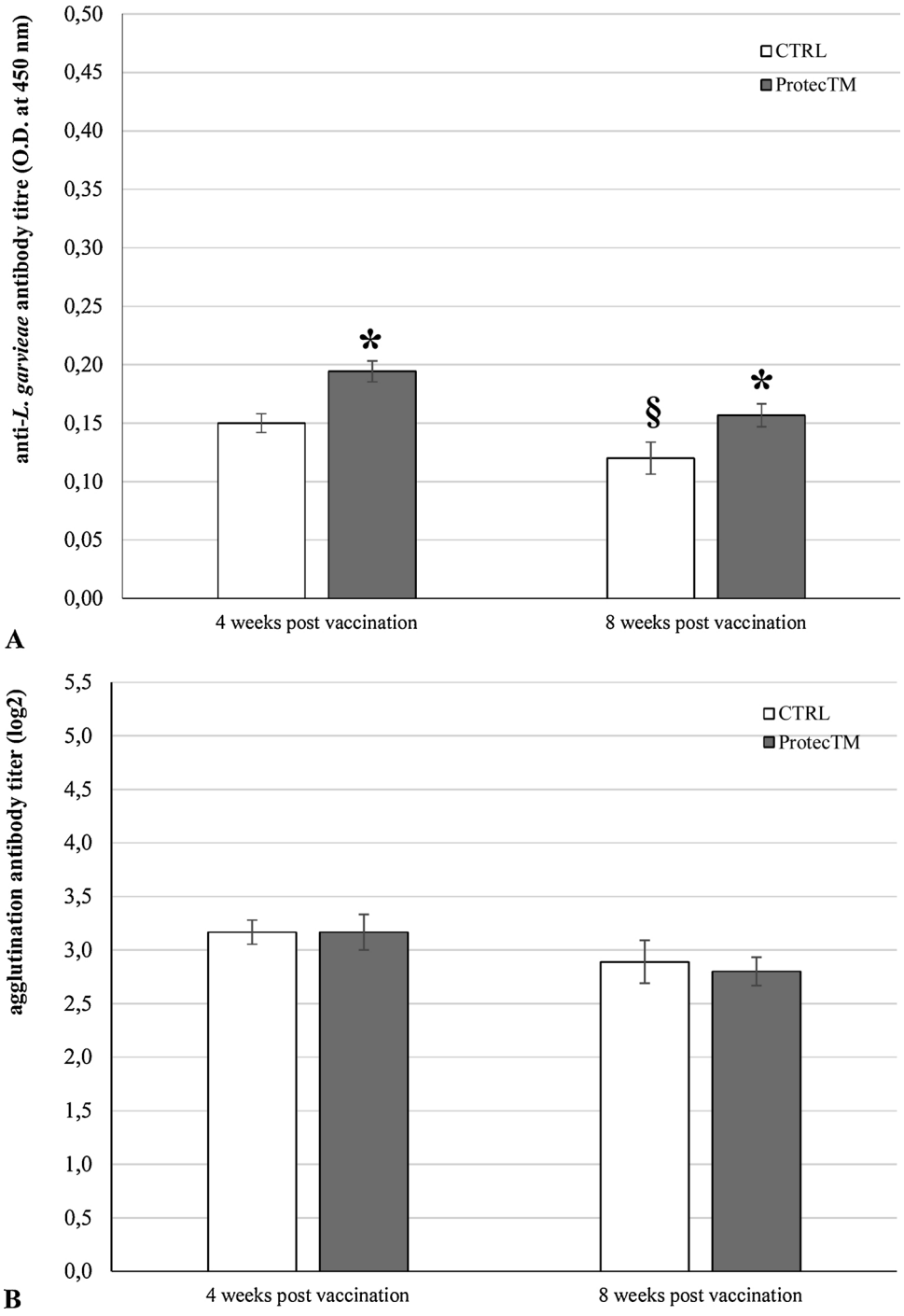

Fig. 5. Specific anti-*L. garvieae* IgM detected by indirect ELISA (OD at 450 nm, serum diluted 1:10) (A) and agglutination antibody titre (log_2 of reciprocal of highest serum dilution that give bacterial agglutination) (B) in rainbow trout fed Protec™ or control diet after vaccination against lactococcosis. Data are expressed as mean ± SEM (n = 12 fish). * indicates a significant difference between fish fed Protec™ and fish fed control diet at each sampling time ($P \leq 0.05$). § indicates a significant difference between fish fed Protec™at 4 weeks-sampling time and at 8 weeks-sampling time within the same treatment group ($P \leq 0.05$).

that not all the specific antibodies developed after vaccination are able to agglutinate the *target* bacteria. In general, the capsulated strains of *L. garvieae* (KG-) isolated from rainbow trout are less susceptible to the agglutination than KG + phenotypes as suggested by Barnes and Ellis (2004), so further studies will be carried out on the *L. garvieae* strain used in the present study for IgM quantification to elucidate this aspect. On the other hand, the agglutination assay is less sensitive than the ELISA technique (Yoshimizu et al., 1992) although it has frequently been applied by other authors to evaluate the level of antibodies against *L. garvieae* in rainbow trout serum (Ghittino et al., 1995; Barnes et al., 2002; Manfrin et al., 2006; Volpatti et al., 2007; Kubilay et al., 2008).

Bibliographic and field data indicate that the coverage of i.p. vaccination against lactococcosis lasts about 3–4 months, which is extended up to six months in the case of vaccines supplemented with adjuvant (Vendrell et al., 2006). In a previous vaccination trial against lactococcosis in rainbow trout, we observed a significant increase of anti-*L. garvieae* IgM three weeks after the i.p. administration of Lacto-Fish Vax vaccine (Fatro), then the antibody titre decrease at 3 month after the immunization treatment by reaching values similar to those detectable in the control unvaccinated group (Bulfon et al., 2016). In

the present study, we instead found that the specific antibody titre against *L. garvieae* remained higher up to 8 weeks after the vaccination treatment in fish fed Protec™ compared to rainbow trout fed the control diet, suggesting a significant effect of the aquafeed in extending for a longer time the synthesis of specific IgM.

Moreover, the present study confirmed that the i.p. vaccination with Lacto-Fish Vax vaccine stimulates a scarce antibody production (low OD values) in rainbow trout as already measured by Bulfon et al. (2016) using the same formulation and by Volpatti et al. (2011) using an alternative *L. garvieae* bacterin at the concentration of 3×10^9 bacterial cells/fish. This evidence suggests a limited immunogenicity of *L. garvieae* bacterin compared to other formulations developed to prevent alternative bacterial diseases in this fish species. Our findings are also consistent with those reported by Bastardo et al. (2012) in a study aimed at evaluating the efficacy of a bivalent vaccine against *Aeromonas hydrophila* and *L. garvieae* in rainbow trout. In fact, these authors reported a significantly higher level of anti-*L. garvieae* IgM at 15 days post-vaccination compared to that detected in unvaccinated fish, but even in this case the antibody titre was low and returned to the basal control level at 90 days after vaccination. Additional investigations are necessary to understand the reasons for what has been observed.

5. Conclusions

The present research suggests that Protec™ aquafeed is able to improve both innate and adaptive immune response of rainbow trout. In particular, our results seem to be interesting in order to find new strategies to improve the effectiveness of the available fish vaccines. However, the mechanisms underlying the immune-stimulating properties of Protec™ components in fish need to be further explored in future surveys. In particular, new insights would be necessary to better elucidate the interaction between Protec™ components and the vaccine, for example by analysing relevant genes involved in innate ad acquired immune response or the *in vivo* resistance to infections. In the present study, a challenge test was not performed, anyway a certain level of protection in rainbow trout vaccinated and fed with Protec™ compared to fish fed with control diet was directly observed in the field by the farmers during the season subsequent to the vaccination campaign. Moreover, other experiments are required to define the adequate level and duration of Protec™ administration in order to promote an optimal immune response in fish.

Compliance with ethical standards

The number of animals used in this research was kept at a minimum where possible and was consistent with achieving the scientific objectives of the study. Pain and distress were minimised by the use of anaesthesia before sampling and vaccination procedures. Fish handling and all the experimental procedures were conducted in compliance with the Guidelines of the European Union Council (Directive, 2010/63/EU) for the use of laboratory animals.

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