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System Biology

Computational modeling of immune system of the fish for a more effective vaccination in aquaculture.

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Abstract

Motivation: A computational model equipped with the main immunological features of sea bass (*Dicentrarchus labrax L.*) immune system was used to predict a more effective vaccination in fish. The performance of the model was evaluated by using the results of two *in vivo* vaccinations trials against *L. anguillarum* and *P. damselae*. Tests were performed to select the appropriate doses of vaccine and infectious bacteria to set up the model. Simulation outputs were compared with the specific antibody production and the expression of BcR and TcR gene transcripts in spleen. The model has shown a good availability to be used in sea bass and could be implemented for other rout of vaccination and more than two pathogens. The model confirms the suitability of *in silico* methods to optimize the vaccine doses and the immune response to them. This model could be applied to other species to optimize the design of new vaccination treatments of fish in aquaculture.
Availability and Implementation: The method is available at http://www.iac.cnr.it/~filippo/c-immsim/
Contact:nromano@unitus.it
Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

The simulation of the immune system is one of the most innovative approaches in bioinformatics, providing a new tool for immunological research [Celada and Seiden, 1992; Merrill, 1998; Lundegaard *et al*., 2007; Bauer *et al.,* 2009; Rapin *et al*., 2010] with the potential to support the vaccine development process [Castiglione *et al*., 2012a]. In contrast to *in vivo* and *in vitro* experiments, computer simulations are cheap, non invasive, ethical and allow consideration of all the variables of the experiment at the same time, providing valuable information on the processes that occur during an infection [.Pappalardo *et al,* 2010; Bown *et al.,* 2012; Castiglione *et al*., 2012b]. Vaccination process could be effectively reproduced by the use of mathematical models to optimize in terms both of time and boosting the vaccine administrations to reduce as much as possible the risk of side effects in individuals [Motta and Pappalardo, 2013]. To date, *in silico* studies have already been applied in mammals including humans, but never in other classes of vertebrates like fish. Teleosts are important food resource in aquaculture and robust vaccination strategies are needed to limit the use of antibiotics. Despite the long track record of past research [Smith, 1988; Scapigliati *et al.,* 2002; Sommerset *et al.,* 2005; Van Muiswinkel, 2008; Magnadottir, 2010] and the availa-

bility of commercial vaccines, the immunization protocols are still to be optimized [Plant, 2011]. The vaccination is commonly limited to a single administration (or priming) and varying according to the fish species [Ellis, 1997; Le Breton, 2009; Touranzo *et al*., 2009]. Among the bacterial diseases affecting the sea bass (*Dicentrarchus labrax,* L.), vibriosis and photobacteriosis are the most frequent, caused by *L. anguillarum* (La) and *P.damseale* subsp. *piscicida* (Psp), respectively [Touranzo *et al*., 2005]. The innovative vaccination protocols developed by our group against vibriosis, consisted on the administration of a commercial formulation (Shering-Plough) by a double immersion or by a subsequent intraperitoneal injection (i.p.), evidencing significant responses, reaching high relative percentages of survival (RPS) after a challenge with a virulent La strain (accounted for >70% in fish immersion boosted and 100% in i.p. boosted ones) [Galeotti *et al*., 2013; Mosca *et al*., 2014]. In a consequent experimentation, juveniles sea bass were vaccinated with a polyvalent formulation against La and Psp. In this work, it was applied the immunological model C-ImmSim [Bernaschi and Castiglione, 2001; Castiglione *et al*., 2005; Motta *et al*, 2005; Pappalardo *et al*., 2005; Castiglione, 2006] to simulate the response of sea bass immune system to two pathogens. To test the model in the ability to properly reproduce the main immunological parameters and fish survival under different vaccination conditions, the *in vivo* experiments results with single or double pathogens were used to compare with the model results. In detail, the work has been divided into three steps: 1) set up of the optimal vaccine/infectious doses; 2) compare *in silico* and *in vivo* specific immune parameters; 3) compare the cumulative mortality rates during the challenge infections. The present study is really innovative as regard the fish and supports the *in silico* approach as a supplementary tool to optimize the design of vaccination treatments in aquaculture before large-scale application. The adaptation of the model to reproduce the immune response of fish *in vivo* is here discussed.

2 Methods

2.1 *Model description*

To reproduce *in silico* the immunological response induced by the tested vaccines, the C-ImmSim model was used [Bernaschi and Castiglione, 2001]. C-ImmSim, a refined version of the original IMMSIM model [Celada and Seiden, 1992], belongs to the Agent-Based Models (ABM) which allow to reproduce the immunological processes as dynamical systems of interacting cellular and molecular entities [Castiglione and Bernaschi, 2005]. The model is polyclonal and it makes use of binary strings (of length 12, in this study) to represent the binding site of B and T lymphocytes receptors (BcRs and TcRs), Major Histocompatibility Complexes (MHCs) and antigens (Ag), epitopes and peptides [Castiglione, 2006]. It includes the major classes of cells of the lymphoid lineage (T helper lymphocytes, Th; cytotoxic T lymphocytes, Tc; B lymphocytes; antibody-producer plasma cells, PLB) and some of the myeloid lineage, i.e., macrophages (MA) and antigen presenting cells (APC). All these entities interact each other following a set of rules describing the different phases of the recognition and response of the immune system against a pathogen. The model considers phagocytosis, antigen presentation, cytokine release, cell activation from inactive or anergic states to active states, cytotoxicity, and antibody secretion. Cells communicate through receptor binding and cytokines. Two entities interact with a probability that is a function of the *Hamming distance* between the binary strings, called the *affinity potential*. For two strings s and s' this probability is equal to one when all corresponding bits are complementary, that is, when the Hamming distance between s and s' is equal to the bit string length. If *NBIT* is the bit string length and m is the Hamming distance between the two strings, the affinity potential is defined in the range 0,…, NBIT as $v(m) = vc^{(m-NBIT)/(mc-NBIT)}$, for $m \geq mc$ and $v(m) = 0$ for $m < mc$, where $vc \in (0,1)$ is a free parameter which determines the slope of the function, whereas $mc(1/2 < mc < 1)$ is a cut-off (or threshold) value below which no binding is allowed. Interactions are coded as probabilistic rules defining the transition of each cell entity from one state to another. Each interaction requires cell entities to be in a specific state choosing in a set of possible states (e.g., *naïve*, active, resting, duplicating) that is dependent on the cell type. Once this condition is fulfilled, the interaction probability is directly related to the effective level of binding between ligands and receptors. All "biological entities" reside on a lattice and represents a known volume of specific lymphoid organ (mm^3) or blood. The model simulates the innate immunity and an elaborate form of adaptive immunity (including both humoral and cytotoxic immune responses). The adaptive immunity follows the widely accepted "Clonal Selection Theory" that states that the immune response is based on specific clones of B and T lymphocytes that are selected for destruction of the antigens invading the body [Burnet, 1959]. More details on the model are reported in supplementary data.

2.2 *The simulation framework*

To adapt C-ImmSim to the immune system of teleosts a computational framework was built by following the methodology of Motta and Pappalardo, [2013; Pappalardo *et al*., 2016]. The haematological and immunological parameters reported in Table 1 were used to assign the number and the half-life of lymphocytes and other cells per mm^3.

Table 1. Haematological and immunological features of teleost fish

Feature	Value/unit	Reference
Blood Volume Range	200-6000 µl	Klontz,1994
Haematocrit	30-35%	Klontz,1994
Erythrocytes percentage	96.5%	PESCALEX, www.pescalex.org
Leucocytes percentage	3.5%	PESCALEX, www.pescalex.org
Lymphocytes	90% L	PESCALEX, www.pescalex.org
B cells in spleen	30 %	Romano *et al*., 1997a; Dos Santos *et al.*, 2000
T cells in spleen	7-9%	Romano *et al*., 1997a; Dos Santos *et al*., 2000
Macrophages	25%	Romano *et al*., 1998
Half-life of B and T cells	10 days	Romano and Scapigliati, p.c. 2016
Lymphocytes duplication rate	1 day	Scapigliati *et al.*,2002
Ab Half-life	23 days	Klontz, 1994; Lobb and Clem, 1991

(L: leucocytes; p.c.:personal communications).

A 3D lattice was chosen to represent a portion of the spleen, which is regarded as the main secondary lympho-myeloid organ in fish [Romano *et al*., 1997a, b]. The other parameters were obtained from the literature since they were widely tested and used in previous studies [Celada and Seiden, 1992; Bernaschi and Castiglione, 2002; Castiglione *et al*., 2005, 2012b; Castiglione and Bernaschi, 2005]. All of the above mentioned parameters have not been modified during our *in silico* study. Each time step (TS) of the model corresponds to eight hours of real life. Stochastic fluctuations comparable to *in vivo* experimental variability are taken into

account in the model by use of pseudo-random numbers driving all probabilistic events.

The vaccination treatments scheduled in LV (against La O1/O2 serotypes) and LPV (against La O1/O2 serotypes and Psp) with the experimental sea bass groups are reported in Figure 1. We use the same experimental scheme to test the model. The description of the experimental conditions, the immunological analyses and the challenge procedures are available in supplementary data.

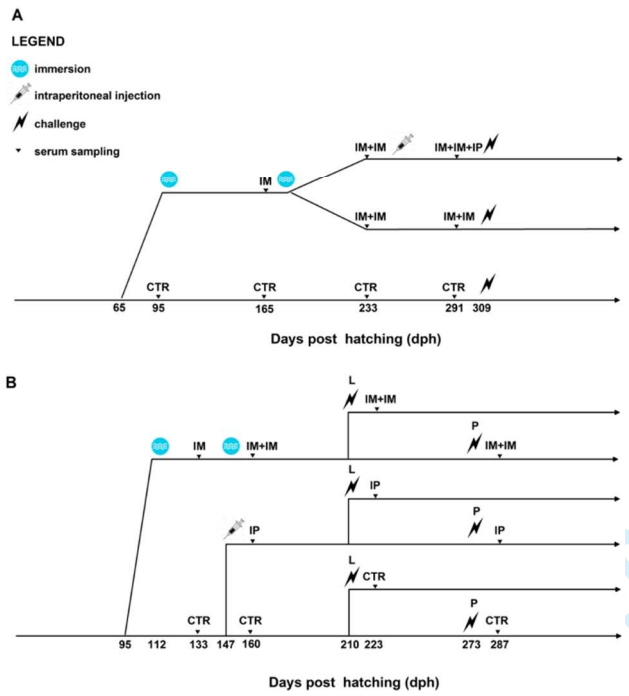

Fig.1. **Scheme of vaccination trials: A) LV vaccination; B) LPV vaccination.**

LV trials consisted of four antigenic injections corresponding to the IM, IM+IM, IM+IM+IP treatments and to the final challenge. Analogously, in LPV trials the antigenic injections were five, corresponding to the IM, IM+IM and IP administrations as well as the challenges with both La and Psp. Further series of tests were carried out to select the appropriate doses of vaccine and infectious bacteria concentrations to be used in the simula-tion experiments. CAL_IP experiment aimed at computing the LD70 for LV and LD50 for LPV, corresponding to the La and Psp suspensions i.p. injected during the *in vivo* challenge procedures (AgCH). To reproduce the activity of the two pathogens the replication factor was set equal to 1.15 for La and 2.3 for Psp, according to the growth curves described in Fernandez-Piquer *et al.* [2011] and Dalgaard *et al.* [2001]. AgCH for La and Psp were calculated by varying the number of antigens, considering those values that produced a survival percentage of 30 and 50, respectively. All the *in silico* experiments consisted on running 100 simulations for each tested value. The *in silico-in vivo* proportional factor α was calculated as the ratio between the computed and the real LD70 and LD50 values. It was then used to compute the number of injected for IM+IM+IP. CAL_IM test was carried out to calculate the number of Ag injected during vaccine immersion (AgIM). In CAL_IM, AgIM was computed by varying the number of Ag absorbed by fish immersion. All simulations were run keeping AgCH constant. The value corresponding to the IM+IM in vivo survival percentage at the dph given from the real experiment was considered. All the *in silico* experiments consisted on running 100 simulations for each tested value. AgCH, AgIM and AgIP values were used to run the simulations for the validation of the model described in the following paragraph.

The vaccination steps were reproduced using the same Ag used in CAL_IP with a replication factor equal to 0. In order to represent the different methods of vaccination tested during the experiments, fish immunization by immersion and i.p. injection have been implemented in this work. In the immersion case, vaccine is introduced homogeneously over the entire grid of calculation, while in the i.p. injection is introduced randomly over the grid. The simulations were stopped at those TS corresponding to 15 days after the challenge phases of LV and LPV trials.

Antibody titres and the number of B and T cells were used to compare *in silico* and *in vivo* data, since they can be considered as the main immunological indicators of both the humoral and the cell-mediated specific immune response [Sunyer, 2013]. In detail, the systematic presence of specific antibodies produced by plasma cells allows to analyse the humoral immunity; the expression of BcR is an indicator of the B cell potential capacity to become plasma cells; the TcRs present in all the T lymphocytes populations are responsible of the cell-mediated specific immune response. In order to fine-tune the simulation results, we conducted a sensitivity analysis (not shown) pointed out on the plasma antibody production rate, the minimum level of molecular affinity among lymphocytes receptors and the level of affinity of Ag peptides to the MHC molecules. Table 1 summarizes the set up of the parameters used to reproduce the vaccination trials. Data were analysed by ANOVA followed by *post hoc* Tukey and Dunnett tests. Homogeneity of variances was tested before data processing. To reproduce *in silico* the mortality curves, the survival percentage was calculated after 15 simulated days for a number of runs equal to the number of fish used *in vivo* for each experimental group. A fish is declared death when the antigen concentration reaches a a defined threshold. Cumulative mortality rates were compared to *in vivo* data. Moreover, the RPS was computed as described in [Amend, 1981], using the same method of *in vivo* challenge analysis.

3 Results

3.1 Optimization of the administered doses

The results of CAL_IP and CAL_IM experiments allow to tune the administration of vaccines to perform LV and LPV simulations (Fig.2). The CAL_IP dose-response curves have similar behaviour as compared with *in vivo* results, and were used to find the LD70 and LD50 in LV and LPV for *in silico* challenges (Fig. 2a). The exponential fit of the curves in CAL_IP gives a high correlation with R^2 equal to 0.998 for La and 0.987 for Psp. To reproduce the vaccination procedure by immersion the results of CAL_IM experiments corresponding to the survival rate of *in vivo* treated fish (Fig. 2b) were used as input. The model shows a lesser accuracy respect to the injection administration, with R^2 values of 0.792, 0.745 and 0.742 obtained for LV trial for LPV ones.

3.2 Comparing in silico vs in vivo immunological parameters

In silico predictions have been tested with *in vivo* results according to the procedure described in section 2.2. Figure 3 shows the comparison between the *in silico* results, expressed as the median on 500 runs for LV and LPV, with the immunological analysis of the specific Ab produced against La and Psp (Fig 3a), BcR (Fig 3b) and TcR (Fig 3c) gene transcripts in spleen during *in vivo* LV and LPV experimental trials. Ab concentrations in LV experiment (Fig 3a.1) show comparable results above all for the IP group, as confirmed by ANOVA ($P<0.001$ on both *in silico* and *in vivo*). Moreover, the humoral response of the model greatly overlap with *in vivo* Ab production even on the rate between the experimental groups titres (up to 50 and 46, respectively). With regards to B and T lymphocytes (Fig 3b.1 and 3c.1), the model response is strongly affected by the i.p. procedure, with values peaking up to 25 and 60 folds right the Ag injection. Overall, the ANOVA results are

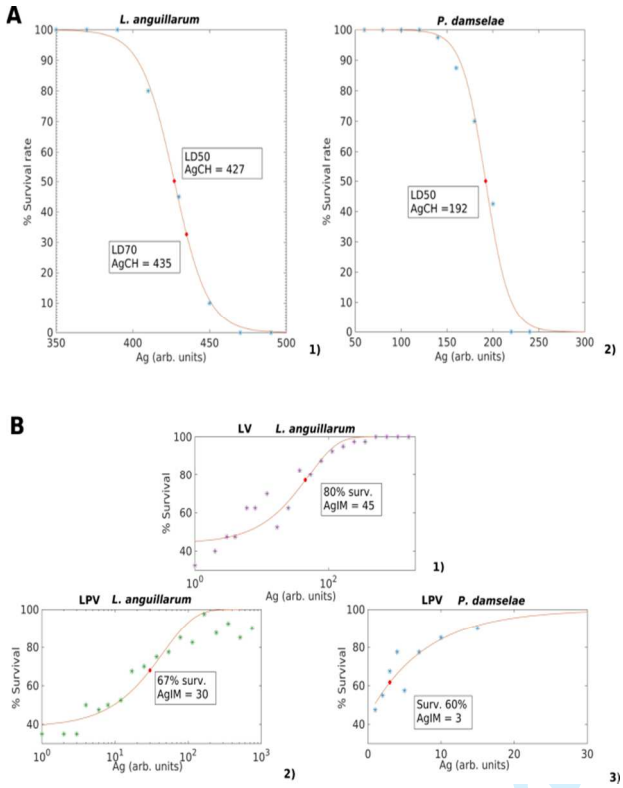

Fig.2 Set up of the optimal doses: A) CAL:IP experiments for 1) La and 2) Psp: the asterisks represent the mean value on 100 runs for each antigen dose; the red points represent the *in silico* data corresponding to the LD50 and LD70 lethal doses used *in vivo*. The results permitted to obtain the Ag variable for the *in vivo-in silico* conversion. B) CAL_IM experiments for 1,2) La and 3) Psp: dose-response curves to obtain the corresponding *in silico* vaccination dose administered by immersion. AgIM is the value which produces a survival percentage comparable for La and Psp in LV and LPV *in vivo* experiments

comparable with *in vivo* ones with P<0.001 for both the groups respect to the controls, for which a great variability of TcR expression in all the groups during the *in vivo* trials were reported. The good performance of the model in simulating LV trial, has prompted us to proceed with the validation by using two different antigens, considering the results of *in vivo* LPV experiments.

Unlike the LV experiment, where the effect of the immunization was detectable only at the subsequent trial step, in LPV the biological essays were computed 21 days after each immunization treatment, allowing the immune system to respond to antigens. For this reason, in LPV the immunization effect is directly appreciable at each vaccination steps.

In the simulations, CTR groups have shown a rapid increase of the immunological variables after the challenge injections, as a result of the immune response activation in surviving fish. The LPV *in vivo* immersion treatment did not increase significantly the production of Ab anti-La like the model did. Instead of the titre of Ab anti-La, the Ab anti Psp in the IM+IM group has greatly incremented after the challenge, reaching comparable values with the IP group. On the other hand, the immunization provided by the i.p. vaccination produced highly specific Ab titres against the two bacteria already from 168 dph (Fig.2a.2-3). Also B and T lymphocytes populations increased already at 168 dph, with maximum values of the T cells for the IP groups and of the B cells for the IM+IM one after both the challenges. In the *in vivo* experiments a substantial increase of both BcR and TcR expression respect to the controls is observed only after the challenge against La.

3.3 In silico vs in vivo survival of fish after bacterial challenges

The comparison between the cumulative mortality recorded after LV and LPV challenges during *in silico* simulations and *in vivo* trials are reported in (Fig.4).

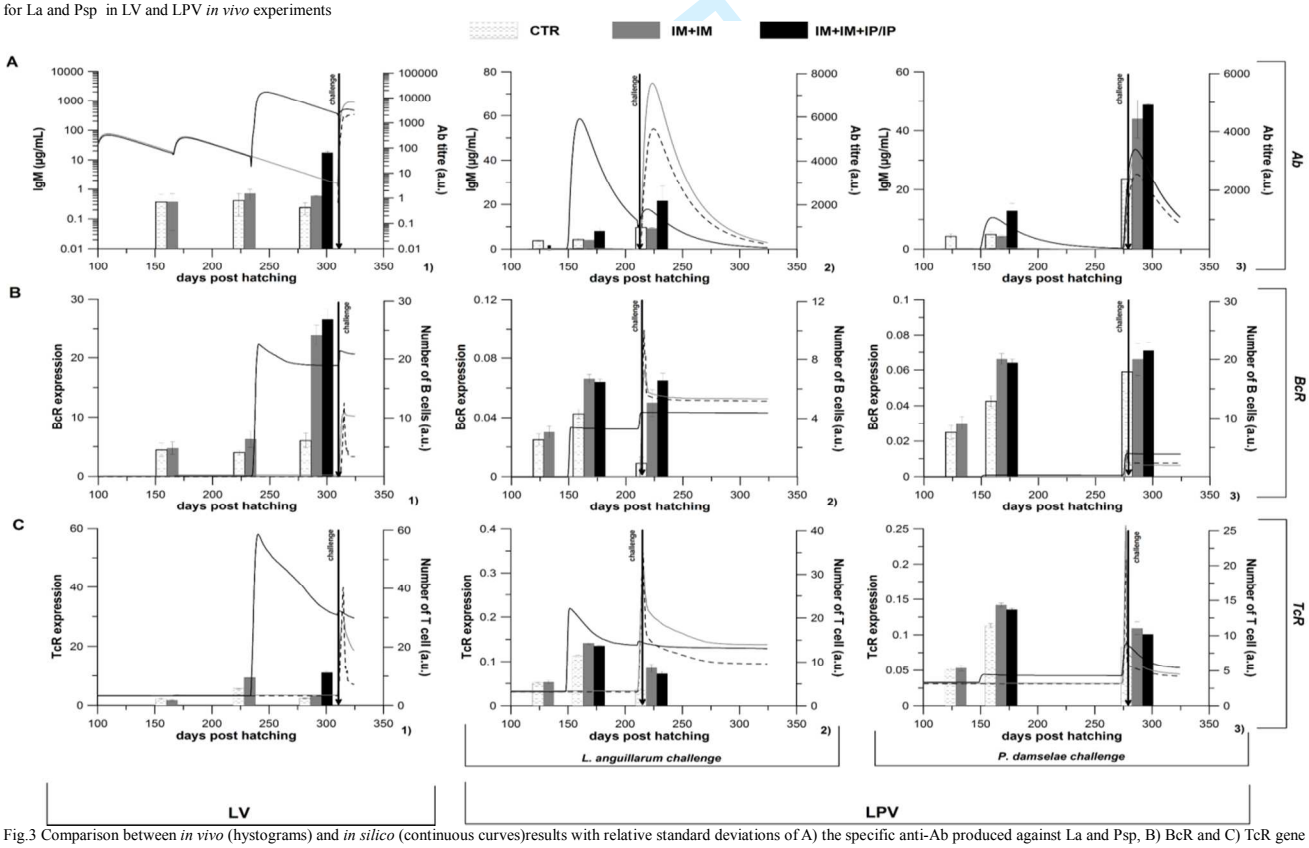

Fig.3 Comparison between *in vivo* (hystograms) and *in silico* (continuous curves)results with relative standard deviations of A) the specific anti-Ab produced against La and Psp, B) BcR and C) TcR gene transcripts in spleen at each scheduled dph of sampling during LV (first column) and LPV trials (second and third columns). The red lines indicate the beginning of challenge infection

In LV simulations, as expected by LD70 injection, the CTR group has reached a percentage of cumulative mortality of 67%, 17% less than the real value. The vaccinated groups highly benefited from the vaccination treatment, almost overlapping with the *in vivo* data (ANOVA, P<0.001 on both *in silico* and *in vivo*). Concerning LPV results, the challenge with La has shown a high correlation (ANOVA P<0.001) with *in vivo* findings: while the i.p. vaccination guarantees a total protection to the pathogen, the cumulative mortality of CTR and IM+IM groups does not significantly differ (35 and 40% respectively, ANOVA P>0.05). Also the challenge with Psp has reported no statistical difference (ANOVA P>0.05) between the CTR and the IM+IM groups (42% and 45%, respectively), as observed *in vivo*. In this case, the IP group has counted a cumulative mortality value of 2%, differing from the *in vivo* result of 31%.

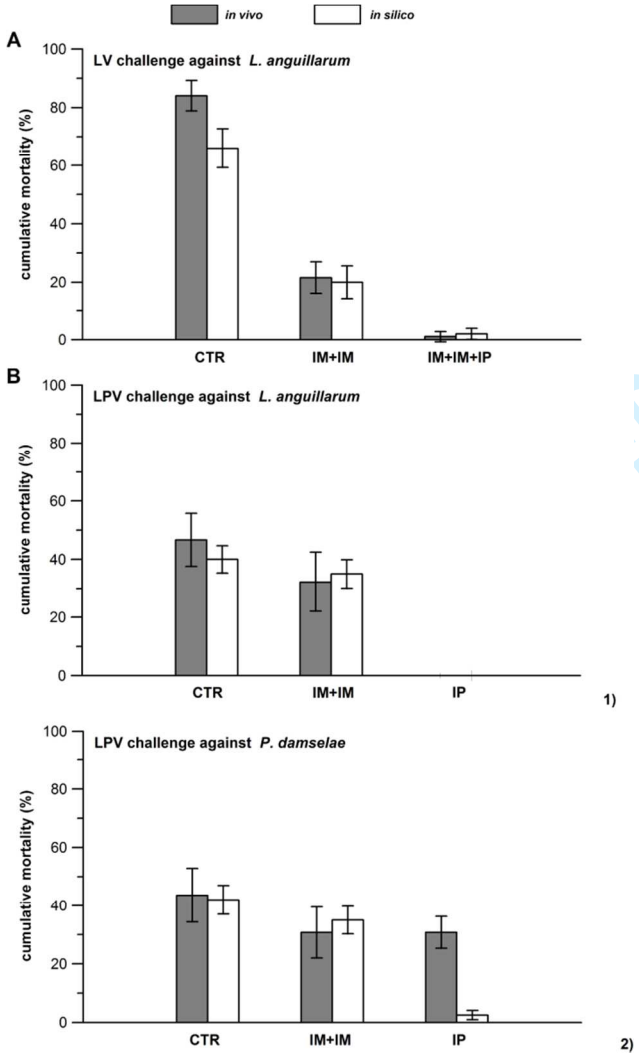

Fig.4 Comparison between *in vivo* and *in silico* cumulative mortality percentages obtained during A) LV and B) LPV trials. In LPV the challenges with La 1) and with Psp 2) are shown.

4 Discussion

Vaccine immersion delivery system is widely used in fish farms, since it is restricted to 100 days post-hatch sea bass and rarely after 6 months [Gudding and van Muiswinkel, 2013; Ellis, 1988]. The advantage is that a large number of small fish can be vaccinated at the same time, even if the antigen uptake is reduced in mucosal tissue [Rombout and Kiron, 2014] as compared to the injection technique [Galeotti *et al*., 2013;Ellis, 1999]. Moreover, the injection is restricted to a considerable size of fish and produces a consistent stress [Lillehaugh, 2014]. In the last years, mathematical models have been widely used in theoretical immunology and vaccinology, enabling epitope discovery for use in rational vaccine design. In several applications over recent years, the C-ImmSim model has generated emergent, sometimes surprising, data that shed light on the mechanisms and interactions of the model itself and on their counterparts in the biological immune system. As an example, during the simulation of the affinity maturation of the humoral response, the varying density of cells and availability of antigen were shown to configure the shift from the severest bottleneck of the primary response, obtaining the T help, to the secondary bottleneck, winning the competition for antigen [Seiden and Celada, 1992]. The use of the model as a computational equivalent of knockout mice or gene transfer in parallel experiments has led to the comparison of the response of the humoral branch only, the cellular branch only, and both branches, to relate the efficiency of responses to different pathogen features[Kohler *et al*., 2000]. In a study about cross-reactive memory, the silencing of one or the other of two suspected kinds of attrition, active or passive, revealed interesting cooperative effects of the combined mechanisms [Selin *et al*., 2004]. In another study, selective "freezing" of humoral cross-reactive responses was obtained by increasing the bit distance in epitopes but not in peptides, while to reveal antibody-mediated competition against cellular responses, the antibody lifetime was artificially shortened or extended over a 50-fold range [Cheng, *et al.,* 2009]. Given its effectiveness in analysing a quite broad range of immunological issues such as host-virus interactions [Castiglione *et al.,* 2005a, b, 2006], hypersensitivity reactions and cancer immunoprevention [Castiglione *et al*., 2003, Motta *et al*., 2005; Pappalardo *et al.,* 2005], the C-ImmSim model was here adapted to reproduce the immune response of sea bass by selecting the specific immunological and haematological features of fish. In particular, the spleen response mechanisms were reproduced, showing memory niches of both B and T lymphocytes [Romano *et al.,* 1997a, b, 1998; Dos Santos *et al*., 2000, 2001]. The first set of tests (CAL_IP and CAL_IM) for the optimization of the doses of antigens (harmless or active) administered by injection or immersion, have reported the typical sigmoid survival trend of biological systems, obtaining a more precise response of the model with the injection procedure. The model well reproduced the pathogenicity of the selected bacteria, needing greater doses of active La respect to Psp to produce a significant mortality (Fig.2a). Differently to La, Psp is an endocellular bacterium able to survive to the oxidative stress due to its capsule. The virulence of this bacteria is also enhanced by the extracellular secretion of proteins, such as proteases, cellulases, phospholipases, hemolysins, and toxins [Rivas *et al.,*2015], which induces the apoptosis of host macrophages [Barnes *et al*., 2005]. The analysis of the immune parameters in LV simulations evidenced a good performance of the model, showing comparable results with *in vivo* experiments. In particular, the model well reproduced the dynamic of the specific Ab titres, that shown a pronounced increase in the i.p. injected group both *in silico* and *in vivo* (ANOVA P<0.001). Concerning the dynamics of B and T leucocytes the model has shown a significant enhancement in the group vaccinated by i.p. injection, not reporting significant responses in the double vaccination by immersion, as reported in *in vivo* experiments, where a great immune response was observed in terms of specific Ab production [Galeotti *et al*., 2013] and TcR and BcR expression both in head kidney and spleen (Romano N., unpublished data). This finding could be related to the great discrepancy between the concentrations of vaccine administered via i.p and immersion and the systemic physiological differences of the antigen uptake [Lillehaugh, 2014]. Furthermore, the model seems to consider all the peritoneum-injected antigens taken and in the same time processed by phagocytes, instead of what happened *in vivo*. The antigen uptake by the macrophages/granulocytes from the peritoneum take at

least two-three days for La and two days for Pdp before to find them in the spleen cells [Folgueira *et al.,* 2015]. The introduction of the depot effect implemented by Castiglione *et al.*[2012a,b], in the model could enhance the performance of the vaccination by intraperitoneal injection, generating a delayed release of the vaccine. *In silico* as well as *in vivo* LPV vaccination performed by using a vaccine containing an inactivated Psp strain combined with two inactivated strains of La has confirmed the effectiveness of the double immersion and i.p. injection procedures in immunizing fish by a significant higher titre of Ab anti-Pdp as compared to that of Ab-anti La. Moreover, the BcR expression in sea bass vaccinated by double immersion has increased at the same level of i.p. injected, being statistically higher respect to the control group already at 168 dph. These *in silico* and *in vivo* results, that demonstrated an efficient coverage in polyvalent vaccination, seem to be in disagreement with several vaccination experiments in the past that did not produced notable results [Dos Santos *et al*., 2001a, b; Romalde *et al*., 2002], probably due to the different trials of vaccination. In fact, the time point to administered the priming and the boosting of vaccine, is really critical in inducing an efficient immune response [Romano *et al*., 2011]. In confirmation of that, a previous double immersion vaccination against *V, harveyi* /Pspin other fish, *S. senegalensis* (5–10 g fish), has induced high levels of protection (>70%) which were similar to those obtained when the respective monovalent vaccines were administered by i.p. route [Arijo *et al.,* 2005]. Interestingly, the results of the simulations has showed that the double immersion procedure does not stimulate immediately the proliferation of T and B memory cells, but is active in the challenge infections. Thus, the simulation seems to predict more effectiveness in double-pathogens vaccination, when is plan three times of administration by immersion instead of two. Conversely, the effect of the i.p. immunization is detectable on both leucocytes populations already before challenge, corresponding to a peak of Ab titer at 168 dph. The model reproduced significantly the post-challenge mortality rates of vaccinated groups in both experiments. Again, the estimation of the immunological coverage ensured by the vaccination against vibriosis is very similar to the *in vivo* results since RPS of fish vaccinated with double immersion was 70% and reached 97% in fish further i.p. boosted, whereas the *in vivo* values were 74% and 99%, respectively. Also the results of the polyvalent vaccination are comparable with the real recorded RPSs, highlighting that the double immersion did not provide a relevant protection as the i.p. treatment did against La (*in vivo* RPS = 33%; *in silico* RPS =35%). The same trend was observed in the challenge against Psp, except for the group vaccinated by i.p. injection, for which RPS reached a percentage of 30%, probably due to the higher time interval elapsed between vaccination and challenge (20 weeks against 11 weeks in the case of La challenge).

In conclusion, the model *in silico* used has shown a great predictive skill on reproducing the survival of fish during a vaccination trial, opening up new horizons in the field of aquaculture research. However, further improvements of the model response are required to fine tune the absorption of the injected antigens. The disparity with *in vivo* dataset could be reduced by inserting specific modules that take into account the adsorption characteristics of the tissues interested by the vaccination procedures (i.e. gills, intestinal tract, peritoneal sac), allowing to set the vaccine administration by oral, or immersion or i.p., or by combining two or all of them. Using an integrated approach between *in vivo* and *in silico* experiments is a cutting-edge innovation in aquaculture, especially in view of the standardization of the vaccine administration, the new environmental sustainability policies and the bioethical containment of use of animals for experiments. In future we will try to test the model by perform firstly an *in silico* experiment (virtual laboratory) and then try to compare the results to *in vivo* experiment by using a small group of animals. Further researches are also addressed to test polyvalent vaccinations against multiple bacteria to provide a standard database for their application also on other fish species farmed in aquaculture.

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Conflict of Interest: none declared.

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